RESISTANCE AND PERSPECTIVES IN SOFT TISSUE SARCOMAS

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Chapter 1

Introduction and scope of the thesis

General introduction

Soft tissue sarcomas (STS) comprise a heterogeneous group of malignant mesenchymal tumors, generally classified according to their resemblance to normal soft tissues. Currently, nineteen histological types and over 50 different subtypes of STS are being recognized.[1,2]

Many of the histological types reveal different biological behavior, but even within a single histological group considerable divergence in the malignant potential has been noticed. During the last decade it has become evident that studies provide more insight when they are specified for histological type and grade.

STS represent 1% of all adult malignancies.[3] Seven percent of all malignancies in children are STS, with rhabdomyosarcoma as the most common type.[4] A major difficulty in the management of STS is the low incidence of the individual histological types, as well as their insidious presentation. This hampers the acquisition of expertise in diagnosis and treatment of these tumors. Therefore, referral to specialized centers is greatly advocated. Careful diagnosis, disease staging and treatment planning by a multidisciplinary team of sarcoma specialists have improved the outcome for STS patients.[5-10] Excellent local control can currently be achieved for most localized STS through surgery, with adjuvant radiotherapy in case of narrow resection margins, high-grade and large tumors. However, patients who are still treated outside specialized centers suffer from higher local recurrence rate and undergo more surgical procedures.[6]

Despite adequate control of the primary tumor, roughly 30-40% of patients progress to metastatic disease. A limited group of patients with pulmonary metastases can be cured with metastasectomy. For the vast majority of patients with disseminated disease, chemotherapy is the only tumor-directed therapy option. Unfortunately, the most active agents doxorubicin and ifosfamide have no curative potential in the metastatic setting. Therefore, knowledge of the mechanisms involved in chemotherapy resistance and the development of new treatment options are crucial for a more effective STS treatment.

One mechanism involved in resistance to chemotherapeutic agents is the expression of proteins by tumor cells that hamper the drugs to reach their target. Expression of P-glycoprotein (P-gp), multidrug resistance-associated protein-1 (MRP1) and lung-resistance related protein (LRP) is associated with cross-resistance to various anticancer agents. Efforts to

inhibit the function of these proteins in order to regain drug activity have largely failed in the clinical situation. Interestingly, a recent study demonstrated that a new generation P-gp/MRP1 inhibitor VX-710 could restore sensitivity to doxorubicin in STS.[11]

An exciting topic in STS treatment is that of targeted drugs with direct access to the apoptotic machinery. Apoptosis is the complex process of cell death, started by an external stimulus and subsequently regulated by intracellular components. Cytokines of the tumor necrosis factor (TNF) family are very interesting, as some members have tremendous potential in inducing apoptosis of tumor cells.[12] Moreover, the prototype TNF-α has already demonstrated its anticancer activity, combined with melphalan, in locally advanced STS in the setting of hyperthermic isolated limb perfusion.[13] Until now, toxic side-effects of TNF-α have prevented it from systemic use. The recently identified tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in its native form appears advantageous, as normal tissues in non-human primates were spared at tumoricidal concentrations.[14] At present, phase I studies with TRAIL are imminent.

A second alternative approach might be the molecular targeted drugs that inhibit receptor tyrosine kinase activity. Aberrant tyrosine kinase activity of tumor cells has been implemented in tumorgenesis and tumor progression. This is illustrated by the malignant gastrointestinal stromal tumors, the most common STS of the gastrointestinal tract, which often bear an activating mutation of exon 11 of the *c-kit* gene. These tumors, while resistant to chemotherapy and radiotherapy, have a high response to the c-KIT inhibitor imatinib mesylate.[15] Current studies focus on whether c-KIT tyrosine kinase might be involved in other STS as well, and on the feasibility of targeting other receptor tyrosine kinases.

The current surgical and radiation treatment of STS is well-defined and further progress with respect to limb saving and local control seems not realistic.[16] The combined modality treatment of surgery and radiation is nowadays focused on diminishing treatment related events and long-term treatment-related morbidity. A better understanding of the tumor biology of STS, together with the development of new systemic treatment may improve of survival of patient with metastasized STS. The studies presented within this thesis were performed to gain insight in mechanisms of drug resistance in STS and to assess potential targets for new treatment options.

Content of the thesis

The aim of the present thesis is:
1. To evaluate mechanisms involved in the biological behavior and responsiveness to chemotherapy on STS.
2. To assess potential targets in STS for new treatment modalities.

Chapter 2 provides a review on factors that are associated with the development of metastases in STS, as this is a major limiting factor in the treatment of STS. Current and evolving opportunities to treat metastatic disease are being discussed.

Chapter 3 presents the results of an immunohistochemical study on P-gp, MRP1 and LRP expression in 141 primary STS separated per histological (sub-)type and grade. Traditionally lumped together, marked differences in biological behavior exist within the group of STS. The results are discussed against the background of these differences.

Chapter 4 outlines the expression of P-gp, MRP1 and LRP in rhabdomyosarcomas, probed by immunohistochemistry. Rhabdomyosarcomas are the most common STS of childhood, but a limited number of cases occurs in adults. While in children the overall survival has been improved since the introduction of chemotherapy next to local treatment, prognosis for adults remains relatively poor. Therefore, it was hypothesized that MDR proteins were being relatively over-expressed in adult rhabdomyosarcomas.

Chapter 5 describes alterations in MDR protein expression in rhabdomyosarcomas after chemotherapy. The results were correlated with the gain in differentiation of rhabdomyosarcoma cells, as mediated by chemotherapy.

Chapter 6 present a study on the expression of MDR proteins in 35 primary STS, compared with that of their matching metastases. This study was performed because despite general impression that metastatic STS is incurable, to date it is still unknown whether metastatic lesions possess more resources of drug resistance.

Chapter 7 addresses the expression of P-gp, MRP1 and LRP in locally advanced STS before and after hyperthermic isolated limb perfusion with tumor necrosis factor-α (TNF-α) and melphalan. In vitro studies demonstrated a modulating effect of TNF-α on these MDR proteins. While TNF-α has been approved for the treatment of locally advanced limb STS, this perfusion setting offered a unique opportunity to translate results from in vitro studies to the actual clinical setting.

Chapter 8 describes the effects of doxorubicin, activated ifosfamide and TRAIL in rhabdomyosarcoma cells. TRAIL alone has demonstrated antitumor activity both in in-vitro and in-vivo experiments, but TRAIL-resistance has been encountered as well. Especially for TRAIL-resistant tumor cells, combination with conventional cytotoxic drugs might be of clinical value.

Chapter 9 reports on a clinicopathological assessment of 16 sarcomas, arising in a previously irradiated area. These so-called postradiation sarcomas are often difficult to treat surgically, while radiation treatment and chemotherapy are not feasible. A previous study mentioned the expression of KIT tyrosine kinase in two postradiation angiosarcomas.[17] With the introduction of the KIT inhibitor *imatinib mesylate* for malignant gastrointestinal stromal tumors, KIT has come into view as a potential target for other tumors as well. Therefore, KIT expression was immunohistochemically assessed in these postradiation sarcomas, including angiosarcomas as well as other histological types. Additionally, the mutational status of exon 11 from the *c-kit* gene was analyzed, as the presence of exon 11 mutations has shown to be correlated to the effect of *imatinib mesylate* in malignant gastrointestinal stromal tumors.[15]

Chapter 10 provides a summary of the studies covered by this thesis with final conclusions and ends with future perspectives in the treatment of STS.

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Chapter 2

Metastasis in soft tissue sarcomas: Prognostic criteria and treatment perspectives

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Abstract

Soft tissue sarcomas (STSs) are rare tumors, notorious for early hematogenous metastasizing. Metastatic disease is seldom amenable to curative treatment; therefore new treatment modalities are required. Treatment-related and tumor-related prognostic factors can be assessed to estimate the risk for subsequent metastases, as will be discussed. By this means, high-risk patients can be defined; they are the candidates for clinical trials mandatory for treatment development. The metastatic process as well as the reaction to chemotherapy depends on the biological make-up of the tumor. Current chemotherapy regimens do not improve the survival rates of patients with metastatic disease, due to resistance mechanisms of tumor cells. New drugs with direct access to the cell death machinery in tumor cells might contribute to more effective treatment of STS patients.

Introduction

Soft tissue sarcomas (STSs) comprise a heterogenous group of malignancies from mesenchymal origin, accounting for approximately 1% of all adult malignancies.[1] STSs tend to metastasize in an early stage, mainly hematogenously with a predilection for the lungs and less frequently metastasize to liver and bone.[2,3] Lymphogenic spread is uncommon (<10%) [4], except for certain histological types such as the rhabdomyosarcomas, synovial sarcomas and epithelioid sarcomas.[5-7] About 10% of patients presents with metastatic disease [4,8-10], whereas almost one quarter of patients with localized disease develop metastases in due course.[11,12] This percentage increases up to 70% for patients with high-grade STSs.[4] The development of metastases poses a major clinical problem, as metastatic disease is seldom amenable to a curative treatment. Moreover, the efficacy of conventional chemotherapy is limited with response rates up to 30% without improvement of overall survival.[13] Eventually, most patients with STS succumb to metastatic disease.[14] Therefore, new systemic treatments are required to improve survival for patients with metastatic disease.

Because of the clinical impact of metastasizing, factors related with the metastatic propensity of STSs are reviewed in this article. A distinction is made between treatment-related and tumor-related aspects, although the two fields can be strongly intertwined. Some of the discussed prognostic

factors are undisputed (e.g. tumor size), while others are still controversial (e.g. surgical margin around a primary tumor).

Clearly there is an increasing awareness amongst oncologists that the biological make-up of the STS is determinative for its response to chemotherapy. Failure to chemotherapy has been linked to several mechanisms, including reduced drug accumulation, alterations in drug targets, increased repair of drug induced cell damage and, and inhibition or defects in the apoptotic routes of tumor cells. The term multidrug resistance (MDR) is used to define the phenotype making tumor cells resistant to functionally and structurally unrelated natural cytotoxic agents. MDR seems to be of particular interest in STS treatment because the 'standard drug' doxorubicin is typically associated with MDR. P-glycoprotein (P-gp) and MDR-associated protein 1 (MRP1) are important mediators of resistance to doxorubicin. The expression of P-gp has been described of prognostic value for several malignancies, including STSs.[15] Ifosfamide is another active agent in STS treatment and is unlike doxorubicin not influenced by P-gp or MRP1. However, it can be inactivated by aldehyde dehydrogenase.[16-19] To what extent this is relevant in treatment failure of STS is unknown.[20]

All anticancer drugs exert their effect by the induction of apoptosis, often by inducing irreparable damage to the DNA. When the apoptotic cascade is not properly executed following damage infliction, tumor cells can become resistant against these drugs. However, tumors cells might then still be sensitive alternative stimuli of apoptosis. In this perspective, molecular targeted drugs with instant access to the apoptotic machinery appear to be of significance. Contrary to conventional agents, these drugs do not damage cell components. Instead, they bind to and thereby activate specific receptors. This switches on apoptosis by subsequent activation of pro-apoptotic factors or inhibition of anti-apoptotic factors. Two classes of molecular targeted drugs with clinical potential in STS treatment will be discussed: the 'death receptor ligands' and inhibitors of receptor tyrosine kinases.

Recent observations suggest that P-gp and MRP1 might act as resistance factors for apoptosis, independently from a drug effluxing function. The possible implications of these phenomena in STS treatment provide the background for a discussion on this issue.

Treatment related aspects of metastasis in STSs

Local treatment

Surgery. Surgery is the mainstay in current STS treatment. In most cases, a local recurrence can be prevented by surgery, with radiotherapy on indication.[21,22] However, despite the frequent success in local tumor control, the risk that distant metastases will develop seems to largely depend on tumor biological characteristics. The following section addresses the impact of surgery on the metastatic process. Furthermore, the role of surgery for metastatic STS lesions is discussed.

Adequate surgical resection of STSs is of the utmost importance in order to minimize the risk for local recurrence. Non-radical surgery will inevitably lead to a local recurrence; nevertheless, radical resection does not warrant the avoidance of local recurrence.[21,23]

The impact of local tumor control on the prevention of systemic disease is complicated to conceive.[24] Trovik et al. evaluated 559 patients with localized extremity or truncal STSs and found that the surgical margin width was not a risk factor for metastases.[25] Contradictory results were reported in a retrospective study of 111 extremity and truncal STSs. A wide tumor-negative margin (10mm or more) was prognostic for a prolonged disease free survival and a reduced rate of distant failure.[26] Analysis of margin status from 2,084 consecutive patients undergoing resection for primary STS showed that positive margins were linked with a significantly slightly higher rate of metastases: 27% *versus* 23% for negative margins.[12] This difference was mainly attributed by the development of metastases after 2 years from surgery for the primary tumor.

Lewis et al. reported on the clinical outcome of a re-resection at Memorial Sloan-Kettering Cancer Center (MSKCC) after non-radical surgery of extremity STSs performed elsewhere.[27] The results were remarkable. Eighty-eight percent of the re-resected patients were disease free after 5 years, compared to 70% of the control group. The occurrence of metastases was lower within the re-resection group, also after correction for risk factors. The possibility of a biasing selection of patients with favorable prognosis into the re-resection group was suggested but could not confirmed by the authors. However, the number of referred patients not eligible for a re-resection, if any, was not mentioned. Such patients might be the ones with an unfavorable prognosis. Another explanation is that the combined treatment of re-resection and adjuvant therapy given at a highly specialized center had a beneficial effect on survival.[28] The results are not

to be considered as a license to freely excise soft tissue lesions suspected to be STSs: injudicious surgical procedures might easily impair the chance for cure.

Local and distant recurrence of STSs are linked events.[29-39] The concept of a causal relation between local recurrence and distant disease has been abandoned. Nowadays, metastasizing of STSs is considered an epiphenomenon independent from local recurrence. In this view, both local and distant failure independently result from an intrinsic, aggressive biological behavior of the primary tumor. Consequently, one might consider high-grade STSs as a manifestation of systemic disease, even when only the primary tumor is detected. While developments in local treatment have reduced local failure rate, distant failure remains unaffected. This is well illustrated by the randomized, prospective study performed at the National Cancer Institute in which limb-sparing surgery was compared to amputation.[21] Patients studied had locally advanced extremity STSs, tumors that are of intermediate or high-grade in most cases.[40] Despite a higher rate of local failure in the limb-sparing group, there was no difference in distant failure and overall survival between the two treatment modalities.[21] Newer limb-salvaging techniques such as the hyperthermic isolated limb perfusion with tumor necrosis factor-alpha and melphalan with or without radiotherapy, have local control rates equivalent to amputation, but do not affect the distant failure rate.[41]

Even when metastatic dissemination has occurred, surgery can be applied with curative intent in selected cases.[42,43] As the majority of patients will develop recurrent lung metastases[42], repeated metastasectomies might contribute to an improved survival in a selected group of patients.[44,45] While only described in a phase 1 setting, an isolated lung perfusion might be of value for selected patients with irresectable pulmonary metastases.[46]

The literature contains few and comparatively small series on surgery for hepatic STS metastases. Chen et al. observed a survival time of 39 months in 11 patients who had undergone radical resection of liver metastases from visceral and retroperitoneal STSs.[47] In addition, Harrison et al. reported on their experience with surgery for liver metastases: in the studied population, 25 STS patients were grouped with patients with other soft tissue lesions.[48] The median survival was 31 months for patients with sarcomas. Only one patient was alive 5 years after surgery. Furthermore, the authors found that tumor grade, tumor type and primary site were not prognostic for survival after hepatic resection. Jacques et al. studied 65 patients with liver metastases from STSs, of which 91% had the primary

tumor at a visceral or retroperitoneal site. A remark to this latter study is that all STSs were diagnosed as leiomyosarcomas, although with current knowledge, part of the visceral tumors might nowadays have been designated as gastrointestinal stromal tumors (GISTs).[49] This has different clinical implications since the introduction of a molecular targeted therapy (see Section *Receptor tyrosine kinase inhibitors*).

While a prospective randomized study in which metastasectomy was compared to no surgery has not been undertaken, the true value of surgery for STS metastases in terms of survival benefit is still undefined. The European Organization for Treatment and Research of Cancer (EORTC) in collaboration with the Scandinavian Sarcoma Group conducted a randomized phase 3 study to elucidate the value of neo-adjuvant chemotherapy followed by pulmonary metastasectomy *versus* metastasectomy alone (EORTC study #62933; available at www.eortc.be (last update January 2002)). Due to insufficient accrual this study has been closed prematurely. However, the aim of the study remains interesting. In conclusion, surgical removal especially of pulmonary metastases is currently the only curative option for patients with metastatic disease resulting in a 5-years survival around 25%. Noteworthy, this option is restricted to a limited group of patients with few pulmonary lesions and a long disease-free survival.

Radiotherapy. Adjuvant radiotherapy after wide local excision is standard treatment for most high-grade STSs.[50,51] With adjuvant radiotherapy, the rate of local and distant recurrence is similar to that of an amputation. External beam radiation is the most frequently applied modality of radiotherapy in STS treatment. The MSKCC is pioneering in the application of brachytherapy, or the interstitial implantation of radioactive material.[52] When technically feasible, brachytherapy offers encouraging results for local control. Brachytherapy has the advantage that when metastases are present, chemotherapy can be applied concurrently.[53] Radiotherapy alone has no role in the curative treatment of STS metastases; however, it does have an important position in the palliation of patients with metastatic disease.[54]

Systemic treatment

Adjuvant chemotherapy aims at the eradication of potential micrometastases in case of a radically removed primary STS without overt metastases. Doxorubicin and ifosfamide are the most active single agents in STS treatment, resulting in response rates up to 25% in patients who did not receive previous chemotherapy.[55] Until now the value of adjuvant

chemotherapy for STSs is not fully elucidated. In most STS studies, the benefit of adjuvant chemotherapy is reflected in an improved disease free survival, but not in an increased overall survival.[56] However, a randomized trial performed by the Italian Sarcoma Group evaluating high-dose doxorubicin and ifosfamide revealed an improved disease free as well as an improved overall survival for patients with high-grade extremity STS.[57] The high-grade extremity STSs were put forward by a previous meta-analysis as the group most likely to benefit from adjuvant chemotherapy.[56]

In case of irresectable metastatic disease, the only treatment directed at the tumor is chemotherapy. Unfortunately, the results of doxorubicin-based chemotherapy for STSs are disappointing: in an analysis of eight randomized studies in this setting, Sawyer and Bramwell report an average response of 21% with very few 5-years survivors.[58] Ifosfamide is also active against STSs, but its current role with respect to survival is limited.[59]

Intrinsic factors of primary STSs influencing metastasis

Since metastasizing forms a great clinical problem in dealing with STSs, it is reasonable that much effort is put in revealing characteristics with supposed predictive value for the development of metastases. Conventional prognostic factors of metastasis are: histological tumor type, tumor grade, tumor size, anatomic site, and tumor stage. These aspects will be detailed below.

Histological tumor type

Despite the great heterogeneity with 19 histological types and over 50 subtypes [14] and new entities still being described, STSs are often considered as a single entity and standard treatment is rather uniform. There is increasing awareness that this is incorrect, as there are marked differences in biological behavior and response to chemotherapy between different histological types.[13]

Metastatic pattern of histological types of STSs. Most types of STS share a predilection for the development of pulmonary metastases, while certain (sub-) types have a different metastatic homing pattern. Malignant GISTs, the most common STS of the gastrointestinal tract and longtime considered as leiomyosarcomas[60], metastasize most often to the liver. However, the true leiomyosarcomas of the gastrointestinal tract mainly metastasize to the lungs.[61] Although rare in other types of STSs,

rhabdomyosarcomas, epithelioid sarcomas, and synovial sarcomas may metastasize lymphogenically.[5-7]

Metastatic propensity of histological types of STSs. For few STS entities, histological typing by itself is helpful to predict the risk for subsequent metastasis: for example, dermatofibrosarcomas protuberans rarely metastasize, whereas the malignant fibrous histiocytomas (MFH) do so as a rule. The histological entity MFH, however, is subject to continuous discussion with respect to its validity, as some authors consider it as a 'waste basket' of different histological types. In fact, subgroups within the group of MFH can be designated that differ in their malignant potential: myogenic differentiation appears to be associated with adverse prognosis.[62] However, for most STSs, especially the poorly differentiated tumors and the unspecified tumors, histological typing is insufficient for prognostic purposes.[63] To give a more accurate estimate, histological grading systems have been developed for STSs (see Section *Histological grade*).

Chemosensitivity of the different histological types. The various histological types of STSs appear to vary substantially in their sensitivity towards conventional and experimental chemotherapeutic drugs. Systemic therapy is effective in improving survival rate only for patients with specific types of STS, even when metastasized. Rhabdomyosarcomas are relatively sensitive to conventional chemotherapy, especially the embryonal subtypes occurring in pediatric patients.[64] Despite its refractoriness to standard doxorubicin-based regimens, the reported response irresectable or metastasized GISTs to a specific molecular targeted therapy is encouraging.[65-67] Metastases from synovial sarcomas may well respond to ifosfamide.[68] Still, the survival seems to depend on a subsequent adequate removal of the tumor remnants.

Histological grade

In order to classify the malignant potential of STSs, several grading systems are in use. The histopathological grading system developed by the members of the French Federation of Cancer Centers (*Fédération Nationale des Centres de Lutte Contre le Cancer, FNCLCC*) is widely used in Europe. Based on differentiation, mitotic count and tumor necrosis [69,70], tumor grade is closely correlated to the future occurrence of metastases.[11,71] Contributing factors of high-grade classification are: few or no morphologic signs of differentiation, a high number of mitotic figures and extensive necrotic areas in a previously untreated specimen.[71] The FNCLCC system encompasses 3 grades, which seems to be more suitable

in predicting metastasis-free survival than a 2-grade system.[72] It has been validated and showed slightly increased ability to predict metastatic recurrence compared to the grading system developed at the National Cancer Institute.[69,71]

Because STSs are rare, grading systems are generally assessed on groups composed of different histological types. However, it is clear that the best approach would be to assess tumor grade per histological type [73], because the elements that define tumor grade have different impact on the various types of STS. For example, quantifying the mitotic activity is important in grading leiomyosarcomas, but not in grading MFH.[14] Moreover, grading systems that define differentiation as the resemblance to normal tissue, can as a matter of fact not be used for STSs without a normal counterpart, e.g. epithelioid sarcomas, alveolar soft part sarcomas and extraskeletal Ewing's sarcomas. In a recent report, Coindre and co-workers studied tumor grade according to the FNCLCC grading system in 1,240 patients presenting with localized disease.[4] This large number allowed for subgroup analysis by histological type and showed that tumor grade was of predictive value for the occurrence of metastases in the commonest types of STSs. It was the most important predictor for MFH, sarcoma NOS and synovial sarcoma, and for a lesser degree for leiomyosarcoma and liposarcoma. In a previous study, Hashimoto et al. studied 1,116 patients, with histological documentation and follow-up information available for 1,005 patients, and reported that histological grade correlated with survival in MFH, leiomyosarcoma and liposarcoma only.[73] Tumor grading –the authors conformed to the guidelines of Enzinger and Weiss [14]– was of no particular value for rhabdomyosarcomas, synovial sarcomas, malignant peripheral nerve sheath tumors and fibrosarcomas. For some of these STSs guidelines are provided, in that they are assigned to pre-set grade.[74] For example, synovial sarcomas, rhabdomyosarcomas and Ewing's sarcomas are considered as high-grade by their clinical aggressive behavior. Nevertheless, Bergh et al. were able to identify subgroups of patients with synovial sarcomas having different risk profiles for developing metastases, based on age, differentiation, and tumor necrosis.[75] Moreover, synovial sarcomas of grade 3 according to the FNCLCC system more often developed metastases than did grade 2 tumors; grade 1 synovial sarcomas were not encountered.[4,76]

Tumor size

STSs can attain large volumes before the establishment of a diagnosis. In an epidemiological outline for the northern Netherlands, 59% of the

primary tumors with documented size had reached a diameter greater than 5 cm.[9] Tumor size is a determinant for both local recurrence and metastatic disease: patients with an extremity STSs larger than 5 cm are significantly more at risk for the development of distant metastases than patients with smaller tumors.[2,11,63] Bauer et al. found that patients with metastases at diagnosis had larger primary tumor than patients with localized disease: median 11 cm *versus* 7 cm, respectively.[8] Patients with a small STS arising in an extremity have an excellent 5-year survival, not influenced by tumor grade. However, when looking beyond the 5-years period as done by Fleming and colleagues, a worse long-term outcome was noticed for small-sized, yet high-grade extremity lesions.[77] Therefore, the authors advocate a prolonged follow-up period for small, high-grade extremity STSs, as 15% of patients developed recurrent disease after 5 years, one-half of which was at a distant location. This is in line with another long-term analysis performed by Lewis et al.[78]

Anatomic site of the primary STS

The site of the primary tumor has great impact on the outcome for the patient with a STS. About one-half of all STSs arise in the extremities, thereby representing the most common site of occurrence.[14] Surgery and radiotherapy provide adequate local control for most of these tumors [21]; still approximately 25% of all patients with extremity STSs develop distant metastases.[79,80] Lungs are by far the most common site to develop metastases.[80] For extremity STSs, deep tumor location is an additional adverse prognostic factor for the occurrence of distant disease.[11] Metastases at the time of diagnosis are found in 3% of subcutaneous lesions, 7% of intramuscular lesions and 14% of extracompartmental lesions.[8] These differences were not attributable to size only, as the authors state that intramuscular and extracompartmental tumors had equal size.

Torosian et al. reported that retroperitoneally and viscerally located sarcomas, accounting for approximately a quarter of all STSs, were more likely to generate distant metastases than tumors from other sites.[81] Linehan et al. demonstrated that even after a radical resection, patients with retroperitoneally or viscerally located liposarcomas had a worse outcome compared to patients with truncal or extremity liposarcomas.[82] Distant metastases were, however, not the major cause for this difference, as a majority of the patients with retroperitoneal and visceral tumors had no evidence of distant recurrence at time of death. A feature that contributes to the lower survival rate of retroperitoneal STSs is the site-dependent difficulty for the surgeon to perform a radical resection. This implies that

the impact of surgery is greater on local control than on preventing distant metastases. Local failure is the most important cause of death for patients with a retroperitoneal STS. Another characteristic of retroperitoneal STSs is their tendency to achieve larger volumes before symptoms become apparent. Large size often implies a more difficult surgical procedure and is in itself an adverse prognostic factor for distant failure (see Section *Tumor size*).

For rhabdomyosarcomas, the primary site has unique considerations compared to other STSs. For example, rhabdomyosarcomas developing in the head and neck region have a favorable outcome, independent of tumor size and notwithstanding their proximity to vital structures.[83]

The site of the primary tumor is linked with the site of distant failure. Metastases from STS of different histological type located at extremities are generally homing to the lungs, while metastases of gastrointestinal STS, mostly GISTs, are predominantly found in the liver.[61]

Tumor stage

STSs are staged by the degree of differentiation together with their local and distant expansion. This information is used for treatment planning and prognostic purposes. Staging of STSs is not uniformly conducted. The two most widely applied staging systems, the one by the American Joined Committee on Cancer (AJCC) and the one by the Musculoskeletal Tumor Society (MSTS) apply different criteria. The AJCC system, now in its fifth edition, is based on size of the primary tumor, lymph node involvement, the presence or absence of distant disease and histological grade based on differentiation.[84] In the MSTS system, tumor stage is defined by (extra-) compartmental expansion, low or high histological grade and evidence of distant metastases.[85] In an attempt to identify the staging system with the best prognostic value on distant metastasis, Wunder et al. studied a group of 300 patients with non-metastasized extremity STS.[86] They applied the fourth and fifth edition of the AJCC system next to the MSTS system, as well as a system developed at MSKCC, which encompasses tumor size, tumor depth and histological grade.[87] The AJCC system proved to be superior to the MSTS and MSKCC system in predicting distant recurrence in this subgroup of STS patients. This implies that when a risk assessment on subsequent metastases is the focus of interest, the latest AJCC staging system would be the best choice.

Resistance of STS to chemotherapy

STSs might initially respond to chemotherapy, but generally resistance develops in due course. MDR is the phenomenon by which tumors are resistant to various structurally and functionally unrelated lipophilic anticancer agents. MDR may be mediated by several mechanisms, including drug transporting proteins and apoptosis inhibitory pathways. These two mechanisms will be discussed in relation to STS treatment, as well as the recent observations that link drug effluxing proteins with impaired apoptosis.

The most widely studied mediator of classical MDR is P-gp, but others drug transporters like the MDR-associated Protein family (e.g. MRP1) and Lung Resistance-related Protein (LRP) are put forward as well. Both P-gp and MRP1 can function as energy-dependent transmembrane pumps that extrude chemotherapeutic agents out of the cell. The exact mechanism of action of LRP is not completely resolved but its causality to MDR has been established.[88] LRP is located intracellularly and may mediate the transport of cytotoxic agents away from their nuclear targets. P-gp, MRP1 and LRP are all three present in a substantial amount of STSs.[40,89,90] It is noteworthy that the standard drug in STS treatment, doxorubicin, is a typical substrate for P-gp and MRP1 and is linked to LRP as well.[91-93]

P-gp, encoded by the MDR1 gene located on the long arm of chromosome 7 (7q21) [94] was first identified in a drug resistant cell line.[95] For STSs, several studies analyzed the relation between P-gp expression and prognosis. The study by Levine et al. in 26 patients with AJCC stage II or III STSs without metastases revealed P-gp expression as an adverse prognostic factor for disease-free survival.[96] Patients with P-gp negative tumors had a median survival more than twice to patients with positive tumors. Interestingly, the prognostic value of P-gp expression seemed to be independent from its role as a mediator of chemoresistance. Nakanishi et al. assessed the P-gp expression in 55 STSs and found high-grade tumors to be significantly more frequently P-gp positive when compared to tumors of intermediate and low-grade malignancy.[97] For intermediate and high-grade STSs, expression of P-gp was found to be an adverse prognostic factor.[97] Therefore, the assessment of P-gp expression might attribute to identify patients at greatest risk for metastasizing within the group of intermediate and high-grade STSs.

Coley and co-workers from London Royal Marsden Hospital assessed P-gp expression in a series of 44 STSs of diverse histology and grade.[98] They found an evenly distribution of P-gp expression over the various

grades, though the authors have not specified the applied grading system. Survival rates did not significantly differ between patients with P-gp positive *versus* patients with negative samples. However, interpretation of the results is restricted by differences in chemotherapy schemes, and only 8 patients received agents that are substrates to P-gp.

A study performed by Jimenez and colleagues demonstrated a correlation between P-gp expression and outcome to chemotherapy in a series of 29 high-grade STSs.[99] Histological grade, ranging from 1 to 4, was based on cellularity, mitotic count, nuclear hyperchromasia, pleomorphism and tumor necrosis; definitions on the grading system are not reported. All 29 patients had grade 3 or 4 tumors and were similarly treated with doxorubicin, dacarbazine and ifosfamide. Ten of the 29 samples (34%) were classified as P-gp positive after detection immunoreactive tumor cells by two antibodies recognizing different epitopes of P-gp. These patients had significantly worse response to chemotherapy in terms of residual viable tumor tissue.

Hijazi et al. assessed P-gp expression in 35 cases of Ewing's sarcoma and peripheral primitive peripheral neuroectodermal tumors.[100] Of the 29 specimens that were obtained before treatment, 16 were P-gp positive. The authors did not find a significant difference in disease free survival between tumors that were negative or positive before treatment, but again the limited numbers of cases hampers reliable conclusions.

In summary, some studies show an increased expression of P-gp in high-grade STSs. However, the clinical implications of P-gp expression remain vague because of small and heterogenous populations of STSs in these studies.

The role of P-gp in STSs of childhood is of special interest. STSs occurring during childhood have a distinct place in that they are mainly rhabdomyosarcomas.[101] In contrast to other types of STSs, chemotherapy is a very important treatment modality in rhabdomyosarcomas, improving survival rates to approximately 70% compared to 30% with surgery/radiotherapy alone.[102] Chan et al. found P-gp as an important adverse prognostic factor in children with STSs.[15] The majority of the patients in this study had a rhabdomyosarcoma; the remaining had undifferentiated sarcomas. Most patients in this study received vincristine, dactinomycin and cyclophosphamide, an efficacious regimen for pediatric STSs.[102] In the group of embryonal rhabdomyosarcomas, an equal number of P-gp positive and negative tumors was present. Significantly more survivors were present in the group of patients with a P-gp negative tumor. However, these results were not confirmed by a later study performed by

Kuttesch et al.[103] However, the studies by Chan et al. and by Kuttesch et al. differed at least partially for the used antigen retrieval method, panel of antibodies, method of immunostaining and scoring of immunoreactivity.

Ifosfamide is a pro-drug that requires biotransformation by the hepatic P450 enzyme system. The active metabolites of ifosfamide can be inactivated within the target cells by the detoxifying enzyme aldehyde dehydrogenase.[104] Alternatively, via the glutathion/glutathion S-transferase (GSH/GST) system, GSH can conjugate to the active metabolites, thereby preventing further cell damage.[105] In STS xenografts, this system proved to be a limited importance.[106]

Alternative pathways of cell death in STS treatment

Anticancer agents exert their effect by inducing apoptosis, or programmed cell death in tumor cells. The damage caused by these agents triggers a complex machinery that completes the disassembly of the tumor cell. Core components of the apoptotic machinery are the so-called caspases, an intracellular cluster of proteolytic enzymes. Upon activation, these caspases cleave specific cellular proteins leading to near-instant cell death. This process is characterized by morphological changes of the cell: plasma membrane blebbing, chromatin condensation, nuclear breakdown and distinctive DNA fragmentation. Eventually, the cell will be decomposed to small apoptotic bodies that are engulfed by neighboring cells without inciting an inflammatory response.

After drug-induced irreparable damage, the cell should be committed to apoptosis in a strictly regulated manner. It has become evident that defects in the apoptotic route can cause resistance to conventional antitumor drugs. Consequently, the clinical efficacy of these drugs will be diminished. An alternative pathway of cell death is provided by molecular targeted agents that have direct access to the apoptotic machinery. Two classes of molecular targeted drugs seem to be of particular value for STS treatment: the death receptor ligands and the receptor tyrosine kinase inhibitors.

Death receptor ligands

Tumor necrosis factor-α (TNF-α*)*, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) are the so-called death receptor ligands, belonging to the TNF superfamily of cytokines. Binding of these ligands to their cognate membrane receptors rapidly induces apoptosis by

activation of the caspases.[107] TNF-α has been demonstrated its efficacy in STS treatment, although limited to the setting of an isolated limb perfusion due its toxicity after systemic administration. Combined with the alkylating agent melphalan, TNF-α leads to an excellent tumor response rate in locally advanced STSs.[108] Because of the loco-regional nature of this treatment modality, it seems reasonable that the rate of metastasizing is not being influenced. Consequently, no improvement in survival is seen. The clinical application of TNF-α could be expanded to metastatic disease if low systemic doses proof to be safe in humans. In rats, combinations of low-dose TNF-α with cytotoxic agents offer a rational approach.[109] Combining low-dose TNF-α with a cytotoxic drug might turn out as a two-sided sword: cytotoxic stress can induce TNF-receptor expression in tumor cells and thereby increase sensitivity to TNF-α. On the other hand, TNF-α has been demonstrated to render tumor cells more sensitive to doxorubicin *in vitro* by modulating P-gp, MRP1 and LRP expression.[110,111] Alternative means of TNF-α administration, like liposomal encapsulation [112] or gene therapy [113] might facilitate a broader application in STS patients.

FasL is cytotoxic for tumor cells, but is also potentially lethal after systemic administration because liver cells are susceptible to its apoptotic action.[114] However, like TNF-α, FasL could be a candidate for local treatment regimens.[115] Alternatively, the FasL/Fas-receptor interaction between neighboring cells appears to be involved in apoptosis induction by several conventional antitumor drugs. This is exemplified by a study of Mitsiades et al. in which doxorubicin was less effective when FasL was cleaved from tumor cells.[116]

TRAIL induces apoptosis in a great variety of tumor cells, while normal mammalian cells are spared, making it an attractive agent for systemic treatment.[117] Cell lines originating from pediatric rhabdomyosarcomas proved to be sensitive to TRAIL even when they were resistant to Fas mediated apoptosis.[118] The safety profile together with its apoptosis-inducing properties in tumor cells make TRAIL an attractive candidate for systemic treatment of STS metastases.

Receptor tyrosine kinase inhibitors

Apoptosis is a default process: apoptosis will emerge when no concurrent, overruling survival signals are present. Many tumor types over-express survival signaling routes, which appears to prevent their termination. Deregulation of these growth factor receptors has been implicated in tumor survival, tumor growth as well as metastasis. Many growth factor receptors are transmembrane proteins with an extracellular

ligand-binding domain and an intracellular tyrosine kinase domain, the so-called receptor tyrosine kinases (RTKs). The general feature of RTKs is activation of tyrosine kinase upon binding of an agonistic ligand to the extracellular part. Oncogenic RTK signaling may be due to autonomous paracrine feedback loops, gene amplification, overexpression and/or mutations. RTK activity might be inhibited by antagonistic antibodies occupying the extracellular domain. However, when activating mutations have occurred, the binding status of the extracellular receptor-part does not regulate the tyrosine kinase activity anymore. An alternative approach is then the inhibition of the intracellular tyrosine kinase by small molecular drugs that readily enter the cell.

c-kit inhibition in STSs. The best evidence of efficacy of small molecular targeted drug in solid tumors is provided by malignant GISTs, the most common mesenchymal malignancy of the gastrointestinal tract. These tumor are characterized by c-kit activation [119], often due to mutations.[120] Physiologically, c-kit tyrosine kinase activity plays a role in proliferation and differentiation of gastrointestinal pacemaker cells, the supposed progenitors of GISTs.[121] Moreover, c-kit protects several cell types from undergoing apoptosis.[122,123] GISTs are notorious for their resistant phenotype against conventional antitumor drugs with a accompanying high expression of MDR conveying proteins.[124] Exposure to STI-571, a small molecular agent targeted at c-kit tyrosine kinase, leads to apoptosis in GIST cells.[125] The first results of clinical trials with STI-571 are encouraging with regard to clinical improvement and tumor response in patients with metastasized GIST.[65,66]

In vitro experiments demonstrate that c-kit confers an anti-apoptotic signal in other STS types as well: activation of c-kit by its natural ligand protects STS cells of neuroectodermal origin against apoptosis.[123]

Other receptor tyrosine kinases a targets for STS treatment. Structurally related to c-kit are the platelet-derived growth factor receptors (PDGF-R), that are like-wise inhibited by STI-571.[126] PDGF-R are widely expressed in mesenchymal cells [127,128], potentially contributing to tumor growth.[129] *In vitro* experiments have demonstrated a potential clinical value of PDGF-R inhibition by STI-571 in dermatofibrosarcoma protuberans.[130] The majority of this type STS mainly causes local problems. However, rare high-grade variants of dermatofibrosarcoma protuberans exist that readily metastasize and therefore may require systemic therapy. In this regard, inhibition of PDGF-R activity leading to apoptosis in dermatofibrosarcoma protuberans cells *in vitro* seems promising.[130] It is questionable whether STI-571 will elicit similar responses in other types of STSs than the GISTs.

Nevertheless, combination with conventional drugs might offer new options in non-GISTs sarcomas. STI-571 combined with doxorubicin results in an synergistic kill of RMS cells *in vitro*.[131]

Other RTK might serve as targets for STS treatment as well. Vascular endothelial growth factor-receptor plays a role for angiosarcoma.[132] A feedback loop involving the insulin-like growth factor is engaged in the formation of rhabdomyosarcomas.[133-135] Inhibition of insulin-like growth factor receptor results in a decreased proliferation and even apoptosis of rhabdomyosarcomas cells *in vitro*.[136] In synovial sarcomas, insulin-like growth factor-1 receptor positive primary tumors were associated with more lung metastases than the negative tumors.[137] Furthermore, its expression was associated with a higher proliferative activity, indicating a higher malignant potential.[138-141]

Expression of functional members of the HER/erbB family of RTKs has been demonstrated in rhabdomyosarcoma cells *in vitro*.[142,143] Modulation of autocrine/paracrine loops that involve these receptors resulted in altered proliferation and differentiation of rhabdomyosarcoma cells. Merlino and Helman reviewed the evidence on RTKs such as fibroblast growth factors and insulin-like growth factor 1 involved in proliferation and differentiation of rhabdomyosarcomas.[144] Further research is required to elucidate the value of these RTKs as targets for treatment of rhabdomyosarcomas and other sarcomas.

P-gp and MRP1 expression protect against apoptosis

P-gp seems to be involved in multiple cellular processes.[145] Apart from its drug-efflux properties, P-gp can inhibit apoptosis[146], as has been demonstrated in several studies on human tumor cells.[147-151] However, not all studies show similar results.[152] How apoptosis is prevented by P-gp is not known, but several mechanisms may be involved: P-gp can alter intracellular acidity [153], thereby rendering a diminished activity of caspases.[154] In analogy to xenotoxin expulsion, P-gp might also pump components of the apoptotic machinery out of the tumor cells, thus blocking an effective execution of apoptosis. Furthermore, P-gp can interfere with the sphingomyelin–ceramide pathway of apoptosis.[155] Interestingly, these studies demonstrated that the ability of tumor cells to go into apoptosis could be restored by inhibitors of P-gp. A single report has linked MRP1 expression with resistance to apoptosis of neuroblastoma cells.[156]

The clinical use of P-gp or MRP1 inhibitors has been hampered by toxic side-effects and has never met the initial high expectations in cancer treatment. Nevertheless, combinations of different P-gp inhibitors at low concentrations can re-establish apoptotic routes, as demonstrated in the *in vitro* situation.[157] This implements that low dose P-gp blockers might lower the threshold for cells to go into apoptosis. Bramwell et al. assessed the efficacy of P-gp and MRP1 inhibitor biricodar with doxorubicin in 29 patients with progressive disease on anthracycline-based therapy.[158] Stabilization of the disease for at least 12 weeks was observed in seven of 15 evaluable patients with non-GIST, indicating the potential activity of this combination.[159] A randomized study should however be performed to confirm this activity.

MDR has been suggested to be directly involved in the metastatic process.[160] Data from a study by Weinstein et al. support the mechanism of P-gp in facilitating metastasis: colon carcinomas with P-gp positive cells invading the surrounding tissues were more likely to have developed nodal metastases as compared to tumors with negative invasive cells.[161] Experimental studies using cell lines in a mice model show that MDR status is related to metastatic potential.[162-164] In the study by Nokihara et al. the formation of metastases was reduced by pharmacological inhibition of P-gp.[164]

Resistance to apoptosis helps tumor cells to survive each of the steps leading to formation of metastases. Several lines of evidence exist that reveal the role of apoptosis in metastasizing. As demonstrated by Wong et al. in an experimental model, as few as 2% of potentially metastatic tumor cells escape from apoptosis, while inhibition of apoptosis led to an increased metastasis formation.[165] When apoptosis was induced in fibrosarcoma cells, less metastases were seen in an experimental animal model.[166] Verapamil, a P-gp inhibitor, decreased the metastatic potential of breast tumor cells in an experimental mouse model.[167] The fact that especially high-grade STSs are notorious metastasizing tumors, suggests that the metastatic cells have an effective anti-apoptotic shelter to their disposal.

Conclusions

STSs are heterogenous malignancies in terms of histological type, primary site, size, stage and grade. All these factors are linked with the metastatic propensity of the primary tumor. Progress in treatment outcome was

achieved by an interdisciplinary approach at specialized centers. When applied by a skilled team, surgery and radiotherapy generally result in successful local control. However, their contribution to the prevention of distant metastases appears to be modest when the tumor has aggressive biological characteristics. Current systemic therapies for STSs with doxorubicin and/or ifosfamide lead to tumor shrinkage in up to 30% of the cases. However, this is not reflected in an improved survival. Childhood sarcomas, mostly embryonal rhabdomyosarcomas, form an exception as chemotherapy greatly improved overall survival for these patients. STSs in adults appear to possess effective mechanisms of resistance against cytotoxic agents. Amongst these are the drug redistributing and drug inactivating proteins, and defective apoptotic routes. Moreover, recent studies revealed that the drug effluxing proteins P-gp and MRP1 might contribute to an apoptosis-refractory state of tumor cells.

Newer molecular targeted drugs provide an alternative in bringing STS cells into apoptosis. Of these, TNF-α and STI-571 already make part of treatment of selected patients diagnosed with a STSs. Preliminary data with the tyrosine kinase inhibitor STI-571 in GIST patients illustrate a promising approach even in metastatic disease of otherwise refractory tumors.

Current clinical research is more and more focusing on the differences within the heterogenous group of STS, such as histological type, immunophenotype, genetic make-up and metastatic behavior. Insight in the background of these differences will provide progress in STS treatment.

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Chapter 3

Expression of multidrug resistance proteins, P-gp, MRP1 and LRP, in soft tissue sarcomas analysed according to their histological type and grade

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Abstract

The biological behaviour of different histological types and grades of soft tissue sarcomas (STS) varies. This might result in a differing sensitivity to cytotoxic drugs. Cross-resistance to functionally and structurally distinct natural-product drugs, known as multidrug resistance (MDR), is associated with the overexpression of P-glycoprotein (P-gp), multidrug resistance-associated protein1 (MRP1) and lung resistance-related protein (LRP). The purpose of this study was to evaluate the expression of P-gp, MRP1 and LRP in STS according to their histological type and grade.

In 141 chemotherapy-naive STS patients, the expression of the three MDR proteins was detected by immunohistochemistry. Nine histological types were documented. These were 19% grade 1, 34%grade 2 and 47% grade 3 tumours. Expression of P-gp and LRP was observed more frequently than the expression of MRP1 ($P<0.0001$). P-gp expression was most pronounced in malignant fibrous histiocytoma (MFH), but was low in leiomyosarcomas. MRP1 was expressed in most malignant peripheral nerve sheath tumours (MPNST). LRP was strongly expressed in MFH and unspecified sarcomas, but was low in liposarcomas. MRP1 and LRP expression was significantly more common in grades 2 and 3 compared with grade 1 tumours. P-gp expression was correlated with MRP1, especially in grade 3 STS.

In conclusion, P-gp, MRP1 and LRP are expressed in the majority of STS, but this expression varies according to the histological type. MRP1 and LRP, but not P-gp expression, were found to be correlated to tumour grade. MDR might contribute to the observed differences in clinical behaviour within the heterogeneous group of STS.

Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumours of mesenchymal origin. At least 19 distinct histological types and over 50 different subtypes have been recognized.[1] STS account for approximately 1% of all adult malignancies.[2] Because of their relatively low incidence, STS are often considered as a single entity. However, large studies that have allowed subgroup analysis by histological type have revealed considerable differences in their biological behaviour.[3-5] In addition, recent clinical studies indicate that the response to chemotherapy is related to the histological (sub) type. For example, leiomyosarcomas

have a poor response rate, while liposarcomas have a favourable response.[3,4] When the disease metastasises, the outcome after chemotherapy is poorer for patients with malignant fibrous histiocytomas (MFH) than for those with other histological types.[4]

A relevant prognostic parameter for STS is histological grade. According to the system of the French Federation of Cancer Centres Sarcoma Group, histological grade corresponds to the degree of differentiation, mitotic rate and presence of tumour necrosis.[6-8] Patients with high grade tumours are at an increased risk of locally advanced disease and metastasis.

Doxorubicin and ifosfamide have the highest single agent activity in advanced STS, with response rates up to 20–30%.[4,9-11] However, a significant improvement in the survival rate has not been reported after doxorubicin or ifosfamide treatment, either as single agents or in combinations.[12]

Multidrug resistance (MDR), whereby tumour cells are resistant to functionally and structurally unrelated natural-product drugs, may result in a failure to respond to chemotherapy treatment. MDR is associated with an increased expression of P-glycoprotein (P-gp)[13], multidrug resistance-associated protein 1 (MRP1)[14], and lung resistance-related protein (LRP).[15] P-gp and MRP1 confer drug resistance by reducing the intracellular drug accumulation due to an active drug-efflux. The spectrum of drugs expelled by MRP1 and P-gp is similar and includes anthracyclines, vinca-alkaloids and epipodophyllotoxins.[16] LRP is identified as the human major vault protein and may play a role in drug transport between the nucleus and cytoplasm.[17] Thereby, LRP alters the intracellular drug distribution, keeping the drug away from its target. The range of drugs associated with LRP is broader than that associated with P-gp and MRP1 and encompasses non-classical MDR substrates such as melphalan and platinum compounds.[18,19]

Histological type and grade are thus linked to the outcome of patients. For this reason, it seems appropriate to analyse the MDR phenotype in STS separated by type and grade. The current study focuses on the expression of P-gp, MRP1 and LRP according to the histological type and grade of 141 chemotherapy-naive STS patients.

Patients and methods

Criteria for inclusion in the current study were a chemotherapy-naive primary tumour with a histological diagnosis of STS and the availability of paraffin embedded tumour tissue. Cases were retrieved using the computerized .les of the department of Pathology from the University Hospital, Groningen. The selected patients were diagnosed between 1979 and 1999.

The study group consisted of 141 STS, obtained from70 male and 71 female patients (mean age: 48.7 years, median: 50 years, standard deviation: 18.8 years, range: 2–89 years). The histological diagnosis was made on hematoxylin-eosin stained paraffin sections, with or without additional immunohistochemical stains. Tumours were classified according to Enzinger and Weiss.[1] There were 27 leiomyosarcomas (19%), 26 liposarcomas (18%), 18 MFH (13%), 14 rhabdomyosarcomas (10%), 12 synovial sarcomas (9%), 8 malignant peripheral nerve sheath tumours (MPNST) (6%), 6 fibrosarcomas (4%), 14 sarcomas not otherwise specified (NOS) (10%) and 16 other STS (11%). The distribution of histological types of the analysed group of tumours roughly reflects the general incidence of these STS.[20]

STS were graded according to the grading system developed by Coindre and co-workers of the French Federation of Cancer Centers Sarcoma Group.[5,7,8] Additionally, grading was performed using the guidelines of the Association of Directors of Anatomic and Surgical Pathology.[21] These guidelines state that certain types (rhabdomyosarcomas, angiosarcomas) are high grade by definition. Although some have demonstrated that different grades between synovial sarcomas can be identified, this type was graded as 3 in our study. Types that were not graded in our study were epithelioid sarcomas (n=3), clear cell sarcomas (n=2) and alveolar soft part sarcoma (n=1).

Immunohistochemistry. From the available paraffin blocks, those containing the most viable parts of the tumour were selected. Immunohistochemistry was performed as previously described in Ref.[22]. After deparaffinisation, heat-induced epitope retrieval was performed. Samples were incubated with the primary antibody for one hour at room temperature. The following monoclonal antibodies were used: C494 (Signet Laboratories, Dedham MA, USA; dilution 1:200) to P-gp; MRPr1 to MRP1 (dilution 1:15) [23]; and LRP (Transduction Laboratories, Los

Angeles CA, USA; dilution 1:400) to LRP. The staining procedure consisted of an indirect immunoperoxidase method using rabbit anti-mouse (C494, LRP) or rabbit anti-rat (MRPr1) peroxidase conjugated immunoglobulins (Dako, Glostrup, Denmark). Bound peroxidase was developed with diaminobenzidine and hydrogen peroxidase. Samples were counterstained with haematoxylin. Paraffin-embedded liver, lung and colon tissues served as positive controls for P-gp, MRP1 and LRP expression, respectively.

Scoring of immunoreactivity. The expression of P-gp, MRP1 and LRP was independently assessed by 4 observers, without knowledge of the clinical data. P-gp, MRP1 and LRP proteins were studied in adjacent slides. The distribution of P-gp, MRP1 and LRP expression was semi-quantitatively assessed by estimating the proportion of positively stained tumour cells. According to previous studies, samples were considered negative for expression of each of the proteins if less than or equal to 5% of the tumour cells were positive.[24,25] Positively scored samples were categorized using a 1-to-4 scale: 1+ for 6–25%positive tumour cells, 2+ for 26–50% positive tumour cells, 3+ for 51–75% positive tumours cells and 4+ for>75% positive tumour cells.

Statistics. The Wilcoxon signed ranks test was applied to compare the level of expression of the distinctive MDR proteins within the same specimens. The Mann–Whitney U test was used to analyse the differences in MDR expression between the histological types and grades. To quantify the correlation between MDR protein expression, the Spearman's rank test was used. A two-tailed P-value of <0.05 was considered to be significant. Statistical software, Statistical Package for the Social Sciences (SPSS) 10.0 for Windows (SPSS Incorporated, Chicago IL, USA) was used for the statistical analysis.

Results

MDR protein expression in the overall group of STS. Table 1 shows the distribution of scores for P-gp, MRP1 and LRP. One sample was not evaluable for P-gp and five samples were not evaluable for MRP1 because of a lack of representative tumour material. P-gp expression was found in

110 of 140 analysed tumours (79%), MRP1 in 67/136 cases (49%) and LRP in 105/141 cases (74%). In the whole group of STS patients, expression of P-gp and LRP was significantly higher than MRP1 expression (both: P<0.0001). In 136 of 141 tumours (96%), at least one of the three MDR proteins was detected.

Table 1. Scoring of expression for P-gp, MRP1 and LRP in the overall group of STS

	P-gp		MRP1		LRP	
Negative	30	*21%*	69	*51%*	36	*26%*
1+	12	*9%*	14	*10%*	12	*9%*
2+	28	*20%*	16	*12%*	25	*18%*
3+	20	*14%*	14	*10%*	23	*16%*
4+	50	*36%*	23	*17%*	45	*32%*
Total	140		136		141	
Missing	1		5		0	

Negative: ≤ 5% positive tumour cells. 1+: 6-25% positive tumour cells. 2+: 26-50% positive tumour cells. 3+: 51-75% positive tumour cells. 4+: >75% positive tumour cells. (Percentages may not add up to 100% due to rounding of numbers).

For 135 tumours, immunohistochemical results were available for all three MDR proteins. In these 135, expression of all three proteins was found in 41 cases (30%), whereas five tumours (4%) scored negative for P-gp, MRP1 and LRP (two leiomyosarcomas, two myxoid liposarcomas and one synovial sarcoma). Co-expression of P-gp and MRP1 was found in 58/135 STS (43%), co-expression of P-gp and LRP in 79/140 cases (56%), and co-expression of MRP1 and LRP in 51 of the 136 analysed STS (38%). When the semi-quantitative scores (negative, or positive1+ to 4+ were analysed), P-gp expression correlated with MRP1 expression (Spearman's correlation coefficient 0.35; P<0.0001). No significant correlation existed between P-gp and LRP, or between MRP1 and LRP.

MDR protein expression in the major histological types of STS. Figures 1, 2 and 3 show the expression of P-gp, MRP1 and LRP in the different histological types, respectively. Only groups consisting of more than 10 cases are discussed hereafter.

Leiomyosarcomas (n=27). Leiomyosarcomas represented the largest group in the current study. All leiomyosarcomas were located at an extremity; no gastrointestinal leiomyosarcomas were included. Seventeen of 27 samples were P-gp negative (63%), significantly more than any other histological type (P<0.001). Most leiomyosarcomas were negative for MRP1: 16/25 samples (64%). Two samples were not evaluable for MRP1. LRP was widely expressed in the leiomyosarcomas: 24 of 27 samples were positive (89%). Most samples had abundant LRP expression (3+ or4+ in 63% of the cases)

Liposarcomas (n=26). Most liposarcomas were positive for P-gp (22/26;85%), with 27% of the cases in each of the categories of 2+, 3+ and 4+. MRP1 immunoreactivity was present in 10/26 samples (38%), of which five were in the lowest category (1+). Like MRP1, LRP expression was absent in most liposarcomas: 15/26 scored negative (58%).
In addition, liposarcomas were analysed for subtype (well-differentiated, myxoid, round cell and dedifferentiated), as this is associated with biological and clinical behaviour. The distribution of the subtypes was: six well-differentiated, 11 myxoid, two round cell and seven dedifferentiated liposarcomas. It was noted that the number of LRP-negative samples in the overall group of liposarcomas was mainly due to the myxoid subtype, with 10 of 11 samples being negative. P-gp and MRP1 expression did not differ significantly between the subtypes.

MFH (n=18). Most MFH were found to have extensive P-gp expression (72% 3+ or 4+). No samples were negative for P-gp. MRP1 staining was less widespread with 47%of the samples staining negative. Three samples could not be evaluated for MRP1 expression. Like P-gp, LRP expression was abundant: there were no negative samples and 72% of the cases were 3+ or 4+.

Rhabdomyosarcomas (n=14). Widespread P-gp staining was common in the rhabdomyosarcomas: 71% of the tumours were 3+ or 4+. Only one sample completely lacked immunoreactivity towards P-gp. MRP1-negative and -positive samples were evenly distributed: 43% versus 57%. Most positive samples had over half of their tumour cells showing immunoreactivity to the MRP1 antibody. LRP was absent or low in all, but three, samples.

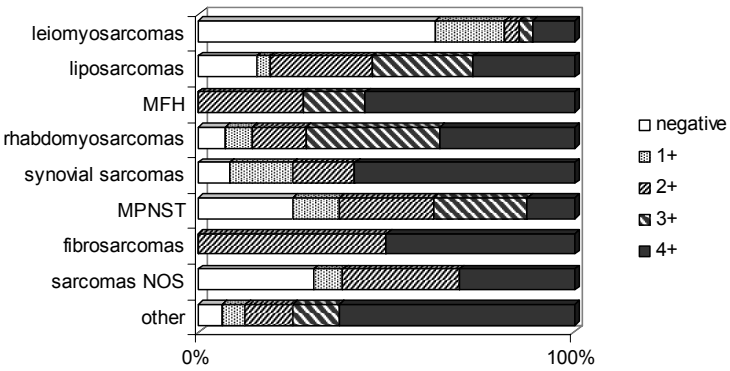

Figure 1. Expression of P-gp per histological type. MFH, malignant fibrous histiocytomas; MPNST, malignant peripheral nerve sheath tumours; NOS, not otherwise specified.

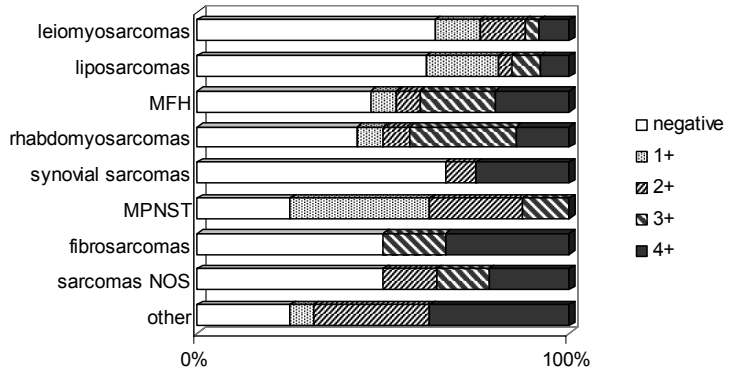

Figure 2. Expression of MRP1 per histological type.

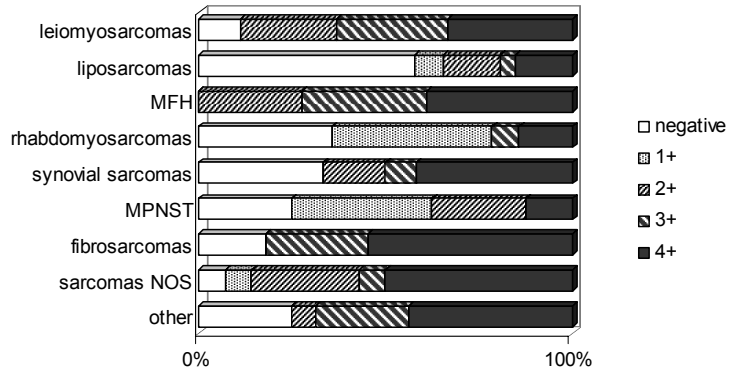

Figure 3. Expression of LRP per histological type.

Like with the liposarcomas, the subtype of rhabdomyosarcoma is associated with varying biological behaviours. In this limited series, no difference in P-gp and MRP1 expression could be observed for the separate subtypes of rhabdomyosarcomas (embryonal, alveolar and pleomorphic). The two samples with >75% P-gp positive tumour cells (4+) were both pleomorphic rhabdomyosarcomas.

Synovial sarcomas (n=12). Fifty-eight percent of the synovial sarcomas had virtually all of their tumour cells showing immunoreactivity to P-gp (4+). In contrast, over 67% of the samples was completely negative for MRP1. The categories of LRP expression were evenly distributed in this group of synovial sarcomas.

Unspecified type (sarcoma NOS; n=14). Amongst sarcomas NOS, a heterogeneous pattern for P-gp expression was observed; one sample was not evaluable for P-gp staining. Fifty percent of the samples were negative for MRP1; the remainder being fairly equally distributed in the 2+, 3+ and 4+ groups. Half of the tumours had the maximal score for LRP staining; only one sample was LRP-negative.

Other types (n=16). The heterogeneous group of rare histological types was lumped together for descriptive reasons. Subtypes included were: angiosarcomas (n=4), epithelioid sarcomas (n=3), clear cell sarcomas (n=2), gastrointestinal stromal tumours (GIST) (n=2), PPNET/Ewing's sarcoma (n=2), malignant haemangiopericytoma (n=1), myxoid chondrosarcoma (n=1), alveolar soft part sarcoma (n=1). This group was characterized by a high P-gp expression (75% 3+ and 4+). No clear pattern was observed for MRP1 and LRP expression.

Histopathological grading of the STS. Of the 141 tumours, 135 were graded according to the aforementioned guidelines. This resulted in 25 grade 1 (19%), 46 grade 2 (34%) and 64 grade 3 STS (47%).

Amongst the grade 1 tumours were 15 liposarcomas (six well-differentiated and nine myxoid liposarcomas), eight leiomyosarcomas, one fibrosarcoma and one malignant haemangiopericytoma. Grade 2 tumours were: leiomyosarcomas (n=14), MFH (n=9); liposarcomas (n=8), MPNST (n=6), sarcoma NOS (n=5), fibrosarcoma (n=2) and other types (n=2). Grade 3 tumours were: rhabdomyosarcomas (n=14), synovial sarcomas (n=12), MFH (n=9), sarcoma NOS (n=9), leiomyosarcomas (n=5), fibrosarcomas (n=3), liposarcomas (n=3), MPNST (n=2) and other types (n=7).

MDR protein expression in relation to histopathological grade. Expression of the evaluated MDR proteins in relation to histopathologic grade is shown in Table 2. P-gp expression was equally distributed over the different grades; however, MRP1 and LRP expression was significantly higher in grades 2 and 3 tumours when compared with grade 1 tumours (P=0.007 for MRP1; P=0.003 for LRP). The correlation between P-gp and MRP1, as observed for the overall group, was more pronounced in the grade 3 tumours with a Spearson's correlation coefficient of 0.54 (P<0.0001).

Co-expression of P-gp and MRP1 (P=0.011), P-gp and LRP (P<0.0001) was significantly more frequently observed in grades 2 and 3 STS compared with grade 1.Co-expression of MRP1 and LRP was not statistically different between grades 2 and 3 STS versus grade 1.

Table 2. Expression of P-gp *(A.)*, MRP1 *(B.)* and LRP *(C.)* in STS according to their histological grade.

(A.)

	P-gp					
	Grade1		Grade 2*		Grade3	
Negative	9	*36%*	12	*27%*	8	13%
1+	2	*8%*	3	*7%*	7	11%
2+	5	*20%*	9	*20%*	13	20%
3+	2	*8%*	9	*20%*	9	14%
4+	7	*28%*	12	*27%*	27	42%

(B.)

	MRP1					
	Grade 1		Grade 2*		Grade 3*	
Negative	18	*72%*	22	*50%*	27	44%
1+	4	*16%*	5	*11%*	4	7%
2+	1	*4%*	7	*16%*	6	10%
3+	1	*4%*	4	*9%*	9	15%
4+	1	*4%*	6	*14%*	15	25%

(C.)

	LRP					
	Grade 1		Grade 2		Grade 3	
Negative	12	*48%*	5	*11%*	18	*28%*
1+	2	*8%*	3	*7%*	7	*11%*
2+	4	*16%*	11	*24%*	9	*14%*
3+	5	*20%*	8	*17%*	10	*16%*
4+	2	*8%*	19	*41%*	20	*36%*

Grading performed according to Trojani, Coindre and co-workers of the French Federation of Cancer Centres Sarcoma Group and using the guidelines of the Association of Directors of Anatomic and Surgical Pathology. Negative: ≤ 5% positive tumour cells. 1+ : 6-25%, 2+ : 26-50%, 3+ : 51-75%, and 4+ : >75% positive tumour cells. (Percentages may not add up to 100% due to rounding of numbers). * Data is missing for some samples.

Discussion

Despite the many different histological types of STS, traditionally they have been lumped together as if they are a single entity. However, marked differences in biological behaviour have underscored the importance of distinguishing the different histological types. Although the response to chemotherapy is rather limited in STS, responses are not strictly uniform, suggesting different mechanisms of resistance in different histological types. The present study evaluated the expression of P-gp, MRP1 and LRP in 141 chemotherapy-naive primary STS patients.

P-gp expression might be associated with a poor response to chemotherapy in childhood rhabdomyosarcomas as well as in adult STS, although the results in different studies are conflicting.[25-29] MRP1 has been detected in STS and co-expression of MRP1 and P-gp expression has been demonstrated to be associated with tumour grade.[30] The combined expression of P-gp, MRP1 and LRP in the various histological types of STS has not been described before.

P-gp expression was found in the vast majority of STS analysed in this study (79%). Previous immunohistochemical studies have reported marked differences in the percentage of P-gp-positive STS, ranging from 0 to 100%.[22,27-29,31,32] These differences can be attributed to methodological variations in the immunohistochemical techniques used (tissue preparation

and storage, epitope retrieval, antibodies used), inclusion of different histological types and limited numbers of samples per group.

Remarkably few leiomyosarcomas expressed P-gp when compared with the other histological types. This appears to contradict earlier reports.[27,30] However, previous studies may well have been confounded by the inclusion of GISTs, as these were previously assumed to be leiomyosarcomas. It has now become evident that GISTs are a biologically and clinically distinct type.[33] GISTs are very chemoresistant, in contrast to true leiomyosarcomas.[34]

A high expression of P-gp in MFH, liposarcomas and synovial sarcomas, observed in previous studies, was confirmed in our study. The criticism with regard to the diagnosis ''MFH'' (some consider MFH a waste basket of several different types) is brought into perspective by a recent study that designated MFH as a distinctive group of STS.[35] This implies that evaluating MFH is still meaningful. MFH have a poor response rate to doxorubicin-based therapy [4], while the current study revealed a substantial expression of MDR proteins, in particular P-gp and LRP. Liposarcomas, that tend to respond better to doxorubicin-based treatment, differed from MFH especially in their expression of LRP.

Although a correlation between P-gp expression and tumour grade was observed in some studies, this could not be confirmed in the present study.[27,30] Nakanishi and colleagues reported a relationship between tumour grade and P-gp expression when comparing high-grade tumours to low and intermediate grade STS.[27] However, a relatively high proportion of MFH (24/55, 44%) were included in their study, which are usually of intermediate or high grade. Therefore, their findings appear to be in concordance with the present study in which all MFH were P-gp positive and had the most extensive P-gp expression. This illustrates again that the relationship between tumour grade and P-gp expression might well depend on the histological types included. Limited data are available on MRP1 expression in STS. In the present group, MRP1 expression was detected in only 49% of cases and co-expression with P-gp was observed in only 43%. Oda and colleagues found co-expression of MRP1 and P-gp mRNA in 38%.[30] In their study, a correlation of tumour grade with the co-expression of P-gp and MRP1 mRNA was found. In the present study, P-gp and MRP1 co-expression was observed significantly more often in the intermediate and high-grade compared with the low-grade STS.

For the overall group, expression of P-gp and MRP1 was moderately correlated, but this correlation became more pronounced when only grade 3 tumours were considered. Furthermore, the present study showed that in

the overall group of STS, MRP1 expression was significantly less frequent than P-gp and LRP expression. Data are scarce on LRP expression in STS. In a comparative study of GIST and leiomyosarcomas, Plaat and colleagues found significantly higher LRP expression in the GIST group.[33] In a study of locally advanced STS, LRP positivity was found in 64% of the cases.[22] Kusakabe and colleagues reported two epithelioid sarcomas were positive for LRP, but not for P-gp or MRP1.[36] Cell lines derived from these epithelioid sarcomas exhibited an MDR phenotype in the absence of P-gp and MRP1, suggesting a role for LRP. One *in vitro* study has demonstrated the presence of LRP in 5 of 7 rhabdomyosarcoma cell lines.[37] In the current study, 74% of the STS were LRP-positive. Expression of LRP was not correlated to P-gp or MRP1 expression. This suggests that the expression of LRP is regulated by factors other than the membrane efflux pumps P-gp and MRP1. Focusing on liposarcomas, all but one of the myxoid subtype were negative for LRP. Myxoid liposarcomas have been previously described to have low or no expression of LRP, possibly due to chromosome breakage near the LRP gene.[38] Liposarcomas other than the myxoid subtype often expressed LRP. The implications of these findings remain speculative, because well-differentiated liposarcomas rarely metastasise and have therefore probably not been included in the study evaluating chemotherapy, whereas other non-myxoid liposarcomas are rare.[1] As the well-differentiated liposarcomas are surgically treated, it is conceivable that MDR has no clear clinical implications for this particular type.

Grades 2 and 3 tumours expressed significantly more MRP1 and LRP than grade 1 tumours. In the recent version of the American Joint Committee on Cancer (AJCC) staging system, grades 2 and 3 are considered together and contribute to patients' prognosis unfavourably.[39] Moreover, co-expression of P-gp and MRP1 and of P-gp and LRP was significantly more often observed in grades 2 and 3 patients. Despite the initial tumour shrinkage observed, especially in high-grade STS, the poor overall survival for patients with high-grade tumours suggests that MDR is a clinically relevant problem. With the observed correlations, MRP1 and LRP expression seem to be indicative of a higher degree of malignancy.

In conclusion, the expression of P-gp, MRP1 and LRP varies between different histological types and grades of STS and it is conceivable that this might contribute to the differences observed in the response to chemotherapy and the outcome of patients with STS.

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Chapter 4

Multidrug resistance proteins in rhabdomyosarcomas: comparison between children and adults

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Abstract

Pediatric rhabdomyosarcomas (RMS) have a more advantageous prognosis after multimodality treatment compared with adult RMS, which might be related to a decreased sensitivity to chemotherapy in adults. Resistance to chemotherapy might be conveyed by the multidrug resistance (MDR)-associated proteins P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and lung resistance-related protein (LRP). It was therefore suggested that these proteins were expressed differently in pediatric and adult patients.

The expression of P-gp, MRP1, and LRP was assessed immunohistochemically in 45 specimens of untreated RMS: 29 were obtained from children younger than 16 years old and 16 were obtained from adults. All children had an embryonal or botryoid RMS. Among the adults, there were 10 embryonal, 3 alveolar, and 3 pleomorphic RMS. Samples were scored as negative or positive according to the percentage of immunoreactive tumor cells: 0.5 (1-5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), or 4 (> 75%).

Expression of LRP was more pronounced in embryonal and pleomorphic RMS in adults compared with RMS in children. In addition, LRP expression correlated with age at diagnosis. Alveolar RMS had remarkably low LRP expression. Expression of P-gp and MRP1 did not differ significantly between children and adults.

In this series of embryonal and pleomorphic RMS, an increased LRP expression was observed in adults, which may explain their worse response to chemotherapy reported in other studies. In alveolar RMS, a low LRP expression was observed, suggesting that other mechanisms are responsible for the resistant phenotype in most of these tumors.

Introduction

Rhabdomyosarcomas (RMS) are a distinct type of soft tissue sarcoma (STS), arising from primitive mesenchymal cells with varying degrees of skeletal muscle differentiation. They are the most common childhood STS, accounting for 3-5% of all pediatric malignancies.[1,2] Approximately 65% of patients are younger than age 6 years when they are diagnosed. Until adolescence, RMS are still among the most common STS, but they become more infrequent with older age and are rarely seen in patients who are older than age 45 years.[1,3,4]

Rhabdomyosarcomas differ from other STS in their favorable response to chemotherapy. Greater than 60% of all patients survive a 5-year period after diagnosis due to multimodality treatment.[5] Factors that determine a patient's prognosis include the site of the primary lesion,[6] stage of disease,[7] histologic subtype,[8] and age at diagnosis.[9-13] Alveolar RMS emerges as genetically and biologically distinct from other subtypes.[14-17] Previous studies comparing adults with children reveal an unfavorable outcome in adult patients.[18-20] In addition, P-glycoprotein (P-gp) expression in tumor cells was an adverse prognostic factor in pediatric patients.[21] Expression of P-gp is associated with multidrug resistance (MDR), a mechanism by which tumors become resistant to a range of structurally and functionally different natural-product cytotoxic compounds.[22,23] Other proteins associated with MDR are multidrug resistance-associated protein 1 (MRP1) [24] and lung-resistance related protein (LRP). Both P-gp and MRP1 act as transmembrane pumps that actively remove toxins from the cell.[25] The spectrum of drugs expelled by P-gp is similar to that of MRP1 and includes anthracyclines, vinca-alkaloids, and epipodophyllotoxins.[26] It is believed that LRP redistributes drugs within the cell, resulting in lower concentrations at the target site.[27] The range of drugs associated with LRP is even broader than those associated with P-gp and MRP1 and additionally includes alkylating agents (e.g., melphalan and cyclophosphamide) and platinum compounds.[28-30] Currently, most children with RMS are treated with a combination of vinca-alkaloids, actinomycin D, alkylating agents (e.g., cyclophosphamide and ifosfamide), and anthracyclines (e.g., doxorubicin).[31,32] When treated with chemotherapy, most adults receive doxorubicin/ifosfamide-based schedules. Therefore, the majority of agents used to treat RMS are associated with MDR.

The current study assesses the relation between the expression of MDR proteins in RMS and age at diagnosis. Although previous studies suggest an unfavorable response to chemotherapy in older patients, it was hypothesized that the expression of MDR proteins is higher within the adult group.

Materials and Methods

Sixty-five patients with newly diagnosed RMS were identified from the computerized files of the Department of Pathology, University Hospital Groningen, The Netherlands. These patients were diagnosed, treated, and/or referred for consultation between 1969 and 2000. Paraffin-

embedded material from the primary tumor was available for 45 patients (70%) who presented between 1979 and 2000. Patient and tumor characteristics were documented for these 45 cases. Treatment modalities are summarized in Table 1. In accordance with clinical practice, patients were considered as adults when diagnosed at age 16 years or older.[33] All RMS were reviewed on hematoxylin and eosin-stained sections with additional desmin immunostains and histologically classified according to Enzinger and Weiss.[1] The sites of the primary tumor were divided into head and neck, extremity, genitourinary (nonbladder/nonprostate), bladder/prostate, and other sites.[34,35] Before treatment, the disease was classified as Stage 1-4 using the TNM staging system developed by the Intergroup Rhabdomyosaroma Study (IRS).[7] This staging system is based on the primary site and tumor size, involvement of regional lymph nodes, and evidence of distant metastases.

Table 1. Treatment and stage of pediatric and adult rhabdomyosarcoma patients.

Characteristics	**Children** *(%)*	**Adults** *(%)*
Treatment		
Chemotherapy alone	6 *(21)*	2 *(13)*
Surgery alone	1 *(3)*	1 *(6)*
Chemotherapy and surgery	11 *(38)*	5 *(31)*
Chemotherapy and radiotherapy	1 *(3)*	1 *(6)*
Surgery and radiotherapy	0	2 *(13)*
Chemotherapy, surgery, and radiotherapy	9 *(31)*	4 *(25)*
Unknown	1 *(3)*	1 *(6)*
Stage*		
1	13 *(45)*	3 *(19)*
2	4 *(14)*	2 *(13)*
3	7 *(24)*	2 *(13)*
4	4 *(14)*	5 *(31)*
Unknown	1 *(3)*	4 *(25)*

* Pretreatment stage according to the Intergroup Rhabdomyosarcoma Study.[7]

Immunohistochemistry. Paraffin-embedded blocks containing the most viable parts of the tumor were selected. Immunohistochemistry was performed using an indirect peroxidase method as described previously.[36] The following monoclonal antibodies were used: C494 to P-gp (Signet Laboratories, Dedham, MA; dilution 1:200); MRPr1 to MRP1 (provided

by Dr. R.J. Scheper, Free University Hospital, Amsterdam, The Netherlands; dilution 1:15); and LRP clone 42 to LRP (Transduction Laboratories, Los Angeles, CA; dilution 1:400). Liver, lung, and colon tissue samples served as positive controls for P-gp, MRP1, and LRP expression, respectively.

Scoring of immunohistochemistry. Two observers, who did not have previous knowledge the clinical data, independently assessed the expression of P-gp, MRP1, and LRP. Expression was categorized as 0, no immunoreactive tumor cells were detected; 0.5, samples contained solitary immunoreactive tumor cells totaling less than 5% of all tumor cells; 1, 5-25% immunoreactive tumor cells; 2, 26-50% immunoreactive tumor cells; 3, 51-75% immunoreactive tumor cells; and 4, greater than75% immunoreactive tumor cells.

Statistical analysis. SPSS for Windows (release 10.0.7; SPSS, Chicago, IL) was used to perform the statistical analysis. To assess differences in MDR expression in samples of the same tumor, the Wilcoxon signed ranks test was applied. To determine a possible association among the expression of P-gp, MRP1, and LRP, the Spearman's rank correlation coefficient (ρ) was calculated. A chi-square test for trend was used to analyze differences in MDR expression between children and adults. A two-tailed P-value of less than 0.05 was significant.

Results

The study group consisted of 29 children (64%) and 16 adults (36%). There was a statistically equal gender spreading within these two groups, with a total of 21 males and 24 females. The median age of the total group was 10 years (range, 0-73 years). The median ages of the children and adults were 4 and 22.5 years, respectively.

Thirty-nine patients had an embryonal RMS (ERMS; 87%), of which 2 were of the botryoid variant. Three patients had an alveolar RMS (ARMS; 7%) and another 3 had a pleomorphic RMS (PRMS; 7%). Figure 1 shows the age distribution with histologic subtype of RMS indicated. All 29 children had an ERMS, including the two botryoid subtypes. Ten adults were diagnosed with ERMS (63%).

Twelve head and neck RMS (27%), 7 extremity RMS (16%), 12 genitourinary RMS (27%), and 2 bladder RMS (4%) were encountered.

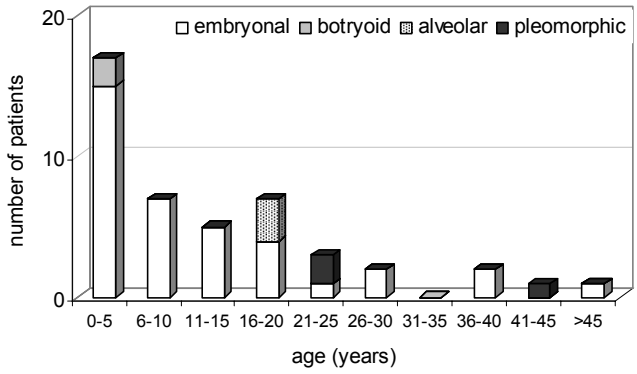


Figure 1. Age distribution of rhabdomyosarcoma patients.

Twelve tumors (27%) were found at other sites. This roughly reflects the distribution found in larger series.[35] None of the categories for primary site was overrepresented in either the pediatric or adult group.

In the preoperative setting, 16 patients had IRS Stage 1 disease (36%), 6 had Stage 2 disease (13%), 9 had Stage 3 disease (20%), and 9 patients had Stage 4 disease (20%). Table 1 summarizes the distribution of disease stage in children and adults. Five adults (31%) had metastatic disease at time of presentation, compared with four children (14%), but this difference was not statistically significant ($P = 0.061$). Data on staging were not accessible in five cases.

MDR protein expression in the overall group of RMS. Most samples (80%) were extensively P-gp positive (scores of 3 and 4), whereas only 2 lacked any reactivity and one showed only minimal expression (a score of 0.5). The majority of the samples (56%) were also extensively positive for MRP1, whereas 5 samples lacked any reactivity and another 5 were only minimally immunoreactive. In contrast, a minority of samples (16%) revealed extensive immunoreactivity for LRP, whereas 12 samples (27%) were completely negative and 7 (16%) showed minimal immunoreactivity (Figure 2).

Expression of P-gp was higher than that of MRP1 and LRP ($P = 0.001$ and $P < 0.0001$, respectively). Expression of MRP1 was higher than that of LRP ($P=0.001$). Expression of P-gp correlated with MRP1 expression:

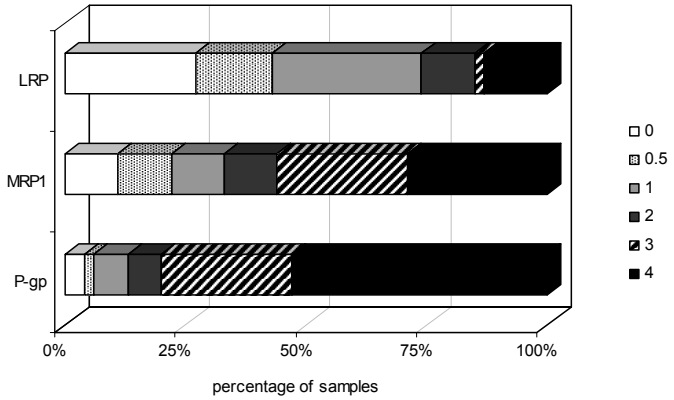


Figure 2. MDR protein expression in rhabdomyosarcomas.

Spearman's $\rho = 0.358$ ($P = 0.009$), whereas LRP expression did not correlate with P-gp or MRP1 expression.

MDR protein expression and histologic type of RMS. Samples that were P-gp negative or sparsely positive (a score of 0.5) were encountered only among the ERMS samples (Table 2). ARMS and PRMS samples had extensive P-gp immunoreactivity (scores of 3 and 4). Samples that were MRP1 negative or sparsely positive also were all of the embryonal type. However, most ERMS samples had extensive MRP1 staining, as was the case for ARMS and PRMS. In contrast, 44% of ERMS samples were scored as negative or sparsely positive for LRP, whereas 33% had limited expression (a score of 1). Two of three ARMS samples were LRP negative, whereas the third sample revealed limited LRP expression. All three PRMS samples had extensive LRP staining.

Table 2. MDR protein expression in histologic subtypes of rhabdomyosarcoma

Level of	Embryonal RMS			Alveolar RMS			Pleomorphic RMS		
immunoreactivity	P-gp	MRP	LRP	P-gp	MRP	LRP	P-gp	MRP	LRP
0	2	5	10	0	0	2	0	0	0
0.5	1	5	7	0	0	0	0	0	0
1	3	4	13	0	1	1	0	0	0
2	2	3	5	1	1	0	0	1	0
3	11	12	1	0	0	0	1	0	0
4	20	10	3	2	1	0	2	2	3
Totals	*(*	*39*	*)*	*(*	*3*	*)*	*(*	*3*	*)*

MDR Protein Expression in Children versus Adults. Figure 3 shows the expression of the MDR proteins in the pediatric and adult samples. Statistical analysis revealed that the expression of P-gp and MRP1 was distributed equally between the pediatric and adult samples. However, LRP expression was more pronounced within the adult group compared with the pediatric group (Fig. 3C). This difference approached statistical significance ($P = 0.052$).

To analyze the confounding effects of other prognostic factors besides age on LRP expression, subgroup analysis was performed. This showed that LRP expression was not associated with disease stage, i.e., Stages 1 and 2 versus Stages 3 and 4 and Stages 1, 2, and 3 versus Stage 4. Subgroup analysis for primary site and histologic subtype was not possible due to the limited number of cases.

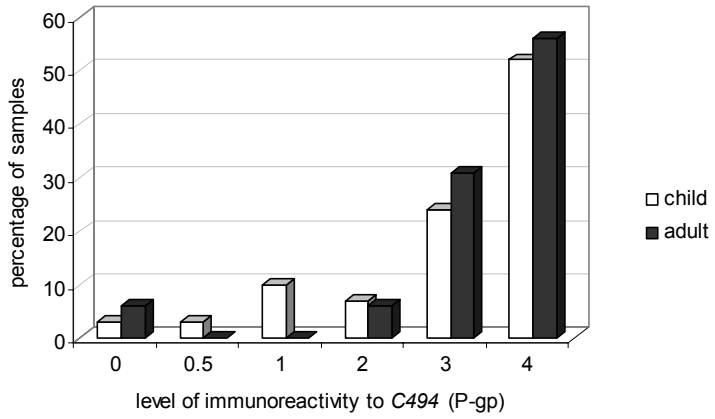
The correlation of expression of the three MDR proteins with patient age (in years) as a continuous variable was also analyzed. Expression of P-gp and MRP1 did not correlate with age at diagnosis. A statistically significant correlation was found for LRP expression with age at diagnosis (in years): Spearman's ρ was 0.341 ($P = 0.022$).

Discussion

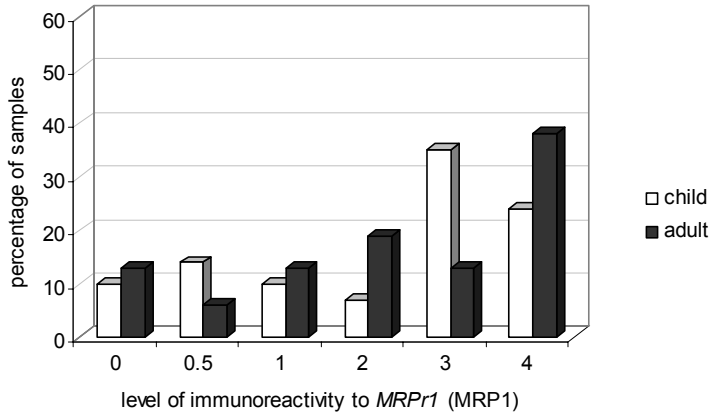
Rhabdomyosarcomas represent a distinct entity within the group of STS, both histopathologically and clinically. They mainly affect children, who have a favorable response to chemotherapy.[5] They may also occur in adults, but previous studies indicated that older patients experience less benefit from multimodality treatment than younger patients. A small number of studies compared prognosis between children and adults,[18-20] whereas others focused on adults only.[3,10,11,33,37]

Several explanations may account for the difference in outcome between pediatric and adult RMS patients. First, adults might present more often in a more advanced stage than children, this being an independent adverse prognostic factor.[5,7] Although children presented more often with IRS Stage 1-3, and more adults presented with IRS Stage 4 disease, the differences were not statistically significant in this study population. Second, in young patients, the primary tumors often arise at sites that are linked with a more favorable response to treatment.[6] For example, patients younger than 10 years often present with favorable genitourinary (nonprostate/nonbladder) or orbital RMS, whereas adults often present with unfavorable limb or retroperitoneal RMS.[38] In our study, a relatively

(A.)



(B.)



(C.)

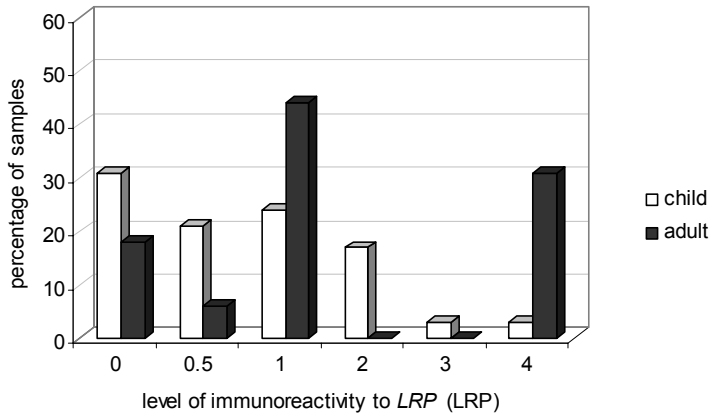


Figure 3. P-gp *(A.)*, MRP1 *(B.)* and LRP *(C.)* in RMS of children and adults.

large number of head and neck tumors occurred in the pediatric group. However, no particular site was clearly overrepresented in either the pediatric or the adult group. Third, patients with ERMS and especially those with the botryoid subtype have a more favorable prognosis than those with ARMS or PRMS.[9,13,39,40] Histologic type and age are associated variables: histologic subtypes are typically clustered around the age of diagnosis.[1,41] Of the current subjects, all children presented with ERMS, whereas the majority of adults were also diagnosed with ERMS (63%). Fourth, treatment-related factors might also affect prognosis. For example, children tolerate relatively higher doses of chemotherapy, potentially allowing more effective multimodality treatment strategies. Due to the heterogeneity with respect to stage and treatment, the outcome of the patients was not evaluated in this relatively small study population.

The main study objective was to analyze differences in MDR protein expression between adult and pediatric RMS patients. Chan et al.[21] found that P-gp expression assessed by immunohistochemistry was an adverse prognostic factor in pediatric RMS patients. Their findings were not confirmed in a later study by Kuttesch et al.[42] However, these two studies differed (at least partially) in the applied antigen retrieval method, the panel of antibodies, the method of immunostaining, and the scoring of immunoreactivity. We found a high percentage of P-gp-positive samples (43 of 45 [96%]) compared with the Chan et al. study (9 of 30 [30%]). This considerable difference might be due to a difference in the immunohistochemical procedure. For example, a heat-induced epitope retrieval was performed in the current study, whereas this was not described in the aforementioned study. In addition, not all specimens in the Chan et al. study were assessed with the C494 antibody, which was used to evaluate all cases in the current study. In the current series, 80% of the samples showed P-gp expression in more than one-half of the tumor cells (Category 3 or 4). The equal distribution of P-gp expression between the pediatric and the adult groups suggests that P-gp is not a critical factor in the clinical behavior between the two groups.

No significant differences were found in MRP1 expression between specimens obtained from children and adults. Therefore, MRP1 expression also does not explain the worse response rate in older RMS patients. The observed correlation between MRP1 and P-gp expression suggests that these drug efflux proteins are regulated by similar factors.

The lung resistance-related protein, LRP, also known as the human major vault protein,[43] is a more recently described structure involved in MDR. Although its exact mechanism in MDR remains elusive, LRP is

believed to relocate the drugs within the cell away from their target. Only a few reports on LRP in RMS are available in the literature. One *in vitro* study demonstrated the presence of LRP mRNA in five of seven RMS cell lines, one of which expressed the actual protein.[44] It is noteworthy that this particular cell line was least sensitive to doxorubicin. The phenomenon of chemotherapy-induced differentiation of RMS cells in postchemotherapy histologic specimens was described by Molenaar et al.[45] A recent report by Klunder et al.[46] demonstrated that differentiation coincided with an increase in LRP expression (but not in the expression of P-gp or MRP1). They suggested that LRP expression enables RMS cells to survive chemotherapy.

Compared with P-gp and MRP1 expression, fewer LRP-positive RMS were encountered. An important finding of the current study is that specimens from adult tumors revealed more tumor cells that were immunoreactive to LRP when compared with pediatric tumors, although this was not statistically significant. The cutoff between children and adults (16 years, based on clinical practice)[33] may appear rather arbitrary from a biologic point of view. Therefore, it is of great interest that LRP expression correlated with age at presentation: Spearman's $\rho = 0.341$ ($P = 0.022$). It is conceivable that LRP expression gradually increases over the total range of patient age.

Increasing knowledge on the genetic and biologic make-up of ARMS indicates that this type is basically distinct from other RMS.[47] The three cases of ARMS in the current study, which all occurred at a young adult age, had either nonexistent or low LRP expression. When statistical analysis was performed after omitting the ARMS, a significantly higher expression of LRP was found in adult tumors compared with pediatric tumors ($P = 0.026$). In addition, excluding ARMS resulted in an increased Spearman's ρ of 0.364 ($P = 0.018$) when calculating the correlation between LRP expression and patient age. However, analysis of this subgroup might have a biasing effect, and multivariate analysis with age and histologic type was not possible due to the limited number of cases. Other mechanisms of drug resistance might be involved in the ARMS than in ERMS and PRMS.

The lack of correlation between LRP expression and P-gp or MRP1 expression suggests that the regulation of LRP is different from that of the membrane efflux pump proteins. Given the rarity of RMS in adults, determining whether LRP expression is clinically relevant in terms of prognosis can only be achieved in multicenter studies in which adult and pediatric patients are treated with uniform chemotherapy protocols.

In conclusion, LRP expression is more pronounced in adult RMS compared with pediatric RMS and is correlated with age. As a genetically unrelated type, ARMS displays no or limited expression of LRP. Larger series of this specific type are required to give definitive answers about the expression and role of LRP. P-glycoprotein and MRP1 are expressed frequently in RMS, but the expression does not differ between pediatric and adult samples. The correlated expression of P-gp and MRP1 suggests that they operate under similar regulatory mechanisms. These findings may contribute to the understanding of the less favorable response of RMS diagnosed with increasing age. Inhibition of LRP function is still in a preclinical stage,[48] but may be an approach to improve the effectiveness of chemotherapy in especially adult, nonalveolar RMS.

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Chapter 5

Expression of multidrug resistance–associated proteins in rhabdomyosarcomas before and after chemotherapy: the relationship between lung resistance–related protein (LRP) and differentiation

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Abstract

Rhabdomyosarcomas generally respond well to chemotherapy, and the residual lesions often are better differentiated than their primaries. This phenomenon may be explained by selective multidrug resistance (MDR) of differentiated tumor cell populations. We assess the role of MDR proteins in chemotherapy-induced differentiation in rhabdomyosarcomas in a clinical setting. Paraffin-embedded samples of 13 pairs of primary untreated rhabdomyosarcomas and their residual, recurrent, or metastatic lesions after chemotherapy were assessed for expression of MDR proteins, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP1) and lung resistance-related protein (LRP). Expression was semi-quantitatively scored based on the percentage of isolated immunoreactive tumor cells as follows: 0, negative; 0.5, <5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%, and 4, >75%. All specimens after chemotherapy, except the late recurrences, were better differentiated than their primary, untreated specimens. P-gp or MRP1 expression did not change significantly, but LRP expression increased significantly after chemotherapy. In both untreated and treated samples, LRP was expressed primarily in differentiated cells. The findings indicate that the *in vivo* expression of LRP, but not of P-gp and MRP1, is induced by chemotherapeutic treatment in rhabdomyosarcomas. The preferential expression of LRP in differentiated cells and the subsequent more extensive expression after chemotherapy suggest that LRP plays a role in therapy-induced differentiation.

Introduction

Rhabdomyosarcomas generally respond fairly well chemotherapy, especially in children.[1-3] The residual lesions often show remarkable morphologic differentiation with increased expression of muscle-specific intermediate filament, desmin, and other myogenic markers.[4-8] Based on comparisons of residual tumors after chemotherapy with their primaries, it has been suggested that chemotherapy selectively eliminates undifferentiated tumor cells and possibly induces further differentiation in the remaining, partially differentiated cells.[8] Studies in rhabdomyosarcoma cell lines have further demonstrated the potential of chemotherapeutic agents to induces differentiation.[9-13]

Response to chemotherapy may be influenced by multidrug resistance (MDR), which reflects the insensitivity of tumor cells to various structurally unrelated natural chemotherapeutic agents. This quality may be already present in chemotherapy-naive tumor cells, and also may be acquired under the pressure of chemotherapeutic treatment.[14,15] A number of proteins have been identified as possibly playing a role in conveying MDR. Among these are the drug efflux pump proteins P-glycoprotein (P-gp) and multidrug resistance–associated protein 1 (MRP1) and the major vault protein lung resistance–related protein (LRP).[14-21] The incomplete response of rhabdomyosarcomas to chemotherapy raises the question as to whether heterogeneity in the expression of MDR proteins plays a role in selective sensitivity of different tumor cell populations. The present study analyzed the expression of these proteins in 13 pairs of primary rhabdomyosarcomas and their residual, metastatic, or recurrent lesions after chemotherapy in pediatric and adult patients.

Patients and Methods

Patients. Cases were selected based on the following criteria: (1) a histologic diagnosis of rhabdomyosarcoma, (2) sufficient paraffin material for both the primary and follow-up specimens, and (3) intervening treatment with chemotherapy. This resulted in 9 pairs of primaries and residual tumors, 2 pairs of primaries and metastases, and 2 pairs of primaries and local recurrences (Table 1). In cases *k* and *l*, the primary tumors were completely resected before chemotherapy (by orchidectomy in case *k* and by resection of a nasal polyp in case *l*), but metastases developed in the retroperitoneum and bone marrow, respectively. In cases *m* and *n*, the primary tumor areas were resected after chemotherapy, but no viable tumor was present. Local recurrences occurred after 17 and 42 months, respectively.

Histology. All cases were reviewed histologically on hematoxylin and eosin-stained sections and with desmin stains. The tumors were further classified as embryonal, alveolar, or pleomorphic according to the criteria specified by Enzinger and Weiss.[22] The relative numbers of primitive undifferentiated cells, of large “rhabdomyoblasts” with ample cytoplasm, and of strap cells with or without cross-striations were compared between primary tumors and the corresponding follow-up material, as was the extent of desmin immunoreactivity.

Table 1. Patient data.

Patient	Age (yrs)/Sex	Site	Type	Primary treatment	Follow-up material	Interval (weeks)
a	13/F	Ovary	E	Chemotherapy[#]	Residual	18
b	16/F	Perineum	E	VAC	Residual	23
c	30/F	Palate	E	VAC+RT	Residual	17
d	40/F	Retroperitoneum	E	C, epirubicin	Residual	15
e	9/F	Retroperitoneum	E	VAC + RT	Residual	18
f	14/M	Tonsil	E	VAC + RT	Residual	30
g	0/M	Nose	E	VAC	Residual	8
h	7/F	Urinary bladder	E	VAC	Residual	17
j	10/M	Retroperitoneum	E	VAC + EVAIA + RT	Residual	27
k	18/M	Spermatic cord	E	CT	Metastasis	58
l	39/F	Nose	E	EVI*	Metastasis	20
m	19/F	Foot	A	EVI	Recurrence	71
n	13/F	Thigh	E	VAC + RT	Recurrence	181

Abbreviations: M, male; F, female; E, embryonal; A, alveolar; VAC, vincristine/ actinomycin D/ cyclophosphamide; EVAIA, etoposide/ vincristine/ actinomycin D/ ifosfamide/ adriamycin; EVI, epirubicin/ vindesine/ ifosfamide; RT, radiotherapy
[#] Further details on chemotherapy not available
* RT on synchronous bone metastases, not on the primary

Expression of multidrug-resistant proteins. P-gp, MRP1, and LRP expression was assessed by immunoperoxidase procedures. Samples were deparaffinated in xylene and alcohol. Antigen retrieval was achieved by heating the samples at 115°C under pressure (10 psi) in an autoclave for 3 cycles of 5 minutes each. The monoclonal C494 (concentration 120 µg/mL, dilution 1:200; Signet Laboratories, Dedham, MA), which recognizes P-gp, was used. For MRP1, noncommercial monoclonal antibody MRPr1 (concentration 20 µg/mL, dilution 1:15; kindly provided by Prof. Dr. R.J. Scheper, Department of Pathology, Free University Medical Center, Amsterdam) was used. For LRP, an anti-LRP monoclonal antibody (concentration 250 µg/mL, dilution 1:400; Transduction Laboratories, Los Angeles, CA) was used. The samples were incubated with the primary antibody-containing dilution at room temperature for 1 hour. For each sample, a peroxidase-conjugated secondary antibody identified the binding of the primary antibody. Diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO) in phosphate-buffered saline was used as the chromagen. Samples were counterstained with hematoxylin. Liver, lung, and colon tissue served as positive controls for

P-gp, MRP1, and LRP expression, respectively. The expression was estimated and scored based on the percentage of single immunoreactive tumor cells as follows: 0, no immunoreactivity; 0.5, <5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, 76% to 100%.

Statistics. The statistical significance of the changes in expression of each MDR protein in tumor pairs was assessed using the Wilcoxon signed-rank test.

Results

Patients. The study group comprises 13 patients (eight pediatric and five adult patients). The relevant clinical data are given in Table 1.

Histology. All except one of the tumors that fulfilled the selection criteria appeared to be of the embryonal type.[22] Only case *m* was of the alveolar type. All cases expressed desmin. Comparing primary tumors and their corresponding residual tumors or metastases, all specimens after chemotherapy showed an increase in differentiation compared with their primaries, as well as increased desmin expression (Figure 1A and B). This was not the case for the late recurrences of cases *m* and *n*.

MDR protein expression. P-gp was expressed in all but one primary tumors and was scored as 3 or 4 in nine of 13 cases. In four cases, including the only negative primary case and both recurring cases, expression increased after chemotherapy. In four cases, P-gp expression decreased, whereas in the remaining five cases, expression was similar in the prechemotherapy and postchemotherapy material (Figure 2A). The changes in P-gp expression were not statistically significant.

MRP1 was expressed in all except two primary tumors and was scored as 3 or 4 in seven cases. In the follow-up material of three cases, including both late recurrences, expression increased strongly after chemotherapy. A few positive cells were found in one metastasis of one negative primary tumor. In five cases, expression decreased, whereas in the remaining four cases, expression remained similar to that before chemotherapy (Figure 2B). The changes in MRP1 expression were not statistically significant.

In contrast to P-gp and MRP1 expression, LRP expression was very limited in the primary tumors and was scored as 4 in only one case. Two cases were negative, and the remaining cases showed only a limited

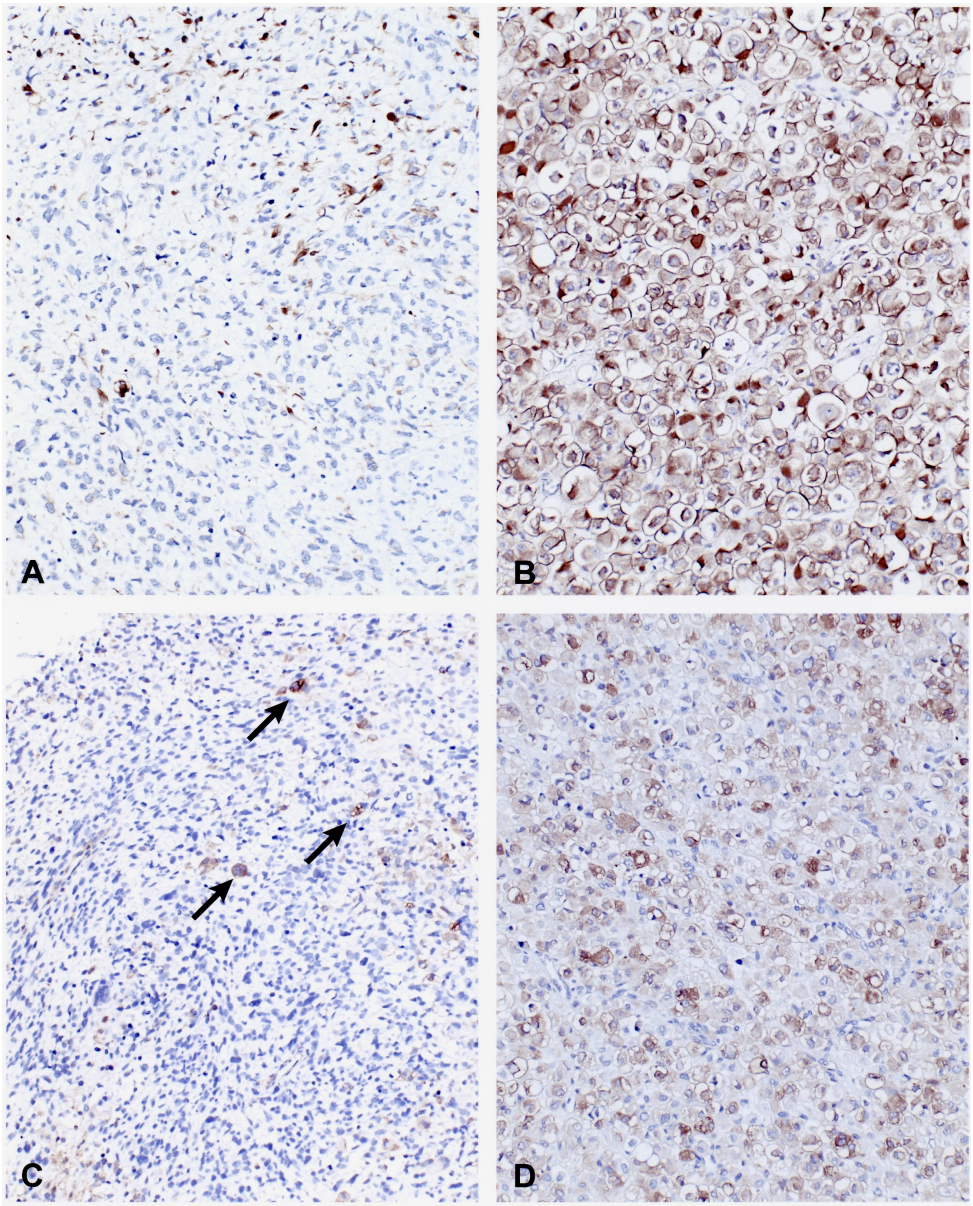


Figure 1. Changes in desmin and LRP expression after chemotherapy. Desmin expression in case *j* before (*A*) and after (*B*) chemotherapy, and LRP expression in case *e* before (*C*) and after (*D*) chemotherapy. Arrows indicate differentiated LRP expressing tumor cells.

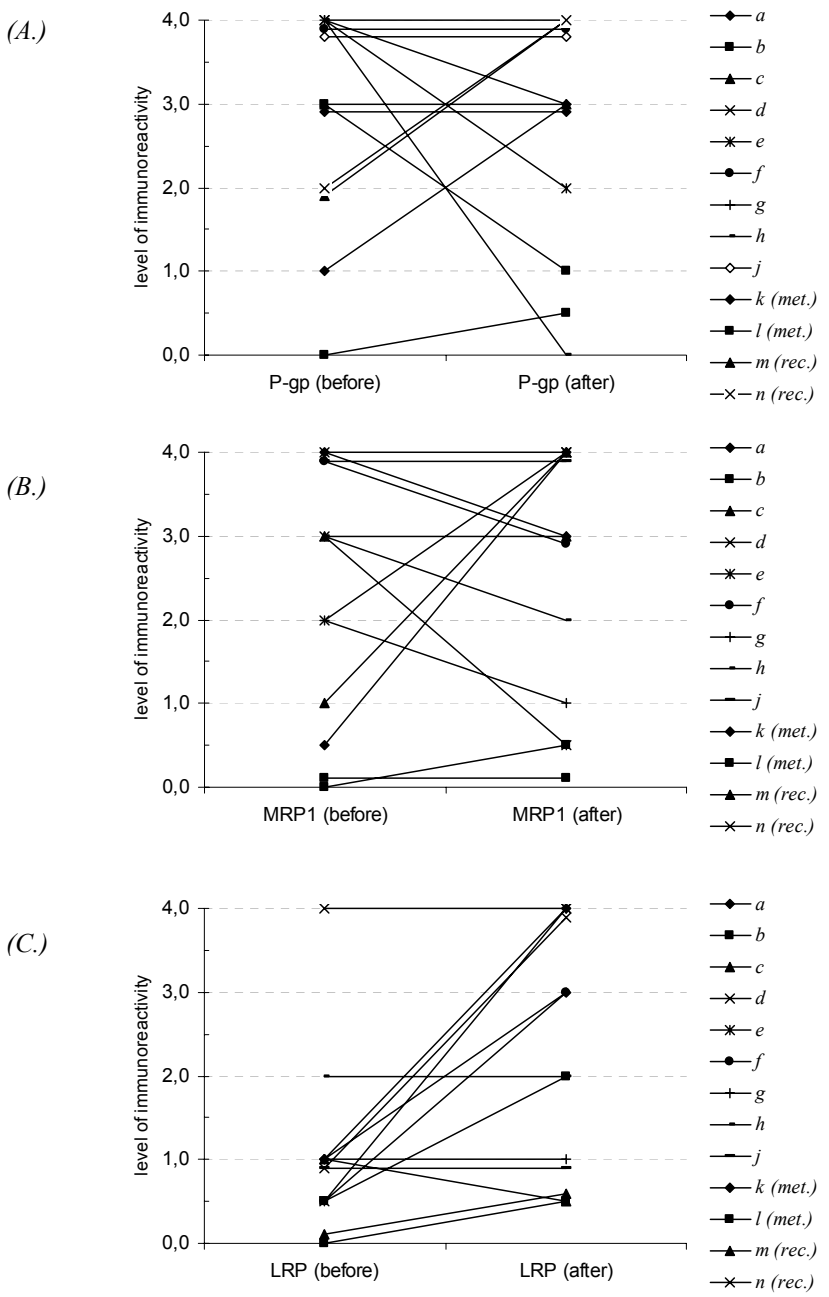


Figure 2. Pairwise comparison of P-gp *(A.)*, MRP1 *(B.)*, and LRP *(C.)* expression in primary rhabdomyosarcomas and their residual lesions, metastases *(met.)* and recurrences *(rec.)* after chemotherapy

number of immunoreactive cells. However, LRP expression was most prominent in better-differentiated tumor cells (Figure 1C and D). After chemotherapy, five of nine residual lesions, both metastases, and one of the two recurrences showed (strongly) increased LRP expression (Figure 2C). Only one residual tumor (case *b*) showed a slight decrease; all of the other postchemotherapy specimens showed expression similar to that of their prechemotherapy counterparts, one of which already showed maximal LRP expression before chemotherapy. The changes in LRP were significant in a pairwise analysis of the whole group ($P = 0.015$), as well as after exclusion of the late recurrences ($P = 0.021$).

Discussion

Chemotherapy-induced differentiation in rhabdomyosarcomas is now a well-established phenomenon, documented in histologic samples [4-8] as well as in cell lines.[9,11,13] However, the mechanisms involved and the clinical significance are still only partially understood. The "differentiated" cells that remain after chemotherapy appear to represent a tumor subpopulation that is resistant to chemotherapy in an otherwise very chemosensitive tumor. This raises the question as to whether MDR may play a role in selective "protection" of better-differentiated tumor cells. To explore this notion, the present study compared the expression of MDR proteins in 13 pairs of primary, untreated rhabdomyosarcomas and follow-up material obtained after chemotherapy. All except two specimens obtained after chemotherapy showed morphologic "maturation" and increased desmin expression, as previously described.[4,7,8] The two cases in which no further differentiation was observed represent late local recurrences in cases in which the primary tumor areas were resected after chemotherapy, but showed complete remission. It may well be that in these cases, primarily chemosensitive clones reemerged.[4,6]

After chemotherapy, P-gp and MRP1 expression remained essentially unchanged, whereas LRP expression increased significantly. These findings suggest P-gp and MRP1 possibly play a role in primary chemoresistance in rhabdomyosarcomas, whereas LRP may be involved in chemotherapy-induced MDR. Studies on chemotherapy-induced changes in MDR in soft tissue sarcomas are rare, and most deal with mRNA, not with protein expression as in the present study. One *in vivo* study demonstrated up-regulation of the mRNA of MDR1 (the gene for P-gp) in five patients after isolated lung perfusion with doxorubicin for lung

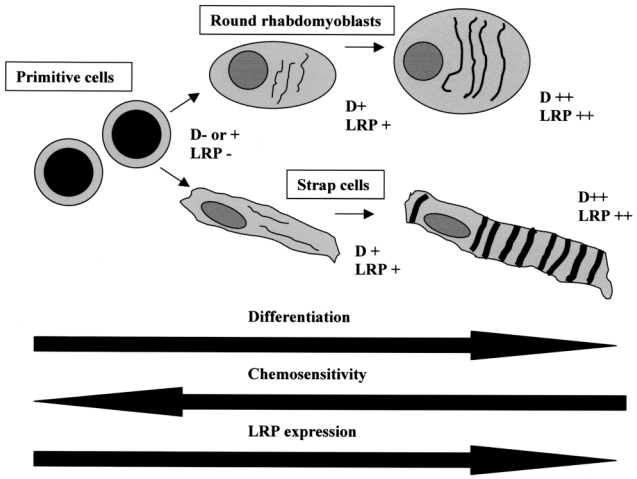
metasases.[23] Other studies have reported higher MDR1 mRNA in treated than in untreated rhabdomyosarcomas, as well as in neuroblastomas and pheochromocytomas [24] and a change from P-gp negative to positive in one sarcoma.[25] The study by Marchal et al. of the embryonal rhabdomyosarcoma cell line RD revealed chemotherapy-induced differentiation not accompanied by up-regulation of MDR1 mRNA.[10] In contrast, another study by the same group showed up-regulation in two rhabdomyosarcoma cell lines, one of which was obtained after chemotherapy *in vivo*.[26] Finally, one study that assessed changes in P-gp, MRP1, and LRP expression in soft tissue sarcomas after isolated limb perfusion with tumor necrosis factor alpha and melphalan found no consistent changes.[27] More data are available on hematologic malignancies. In a series of more than 100 bone marrow biopsy samples from patients with plasma cell myeloma, P-gp was increased after treatment with doxorubicin and vincristine [28]; in both acute myeloid and acute lymphoid leukemias, MRP (but not MDR1) mRNA was increased significantly after treatment [29], and P-gp function and expression were unchanged between diagnosis and relapse in blasts of 30 patients with acute myeloid leukemia.[30] In 17 pairs of bone marrow biopsies of acute myeloid leukemia patients, LRP was increased at relapse compared with the pretreatment specimens.[31] The increased LRP expression was accompanied by morphologic differentiation in the residual specimens. Interestingly, a relation between maturation and expression of major vault proteins (MVPs), the major component of LRP, was also recently established in human dendritic cells.[32] Moreover, blocking of MVPs led to disturbed maturation and decreased viability of these cells. In a study of various tumor types, Izquierdo et al. found that LRP expression was higher in differentiated tumor types and present in a number of refractory tumors after chemotherapy, including two rhabdomyosarcomas.[18] Recent studies by Kitazono demonstrated that LRP mRNA and protein increased after induced differentiation in a colon carcinoma cell line.[33,34]

Although the same phenomenon has been observed in different malignancies, this does not necessarily mean that the same mechanisms are involved. Chemotherapy-induced differentiation of tumor tissue may be explained by selective destruction of undifferentiated tumor cell subpopulations, by direct induction of differentiation, or a combination of both of these mechanisms. Based on morphologic and immunohistologic observations, it has been suggested that both elimination of the most primitive, chemosensitive tumor cells and differentiation induction of committed cells occur.[4,8,9,13] Several studies in rhabdomyosarcoma cell

lines have demonstrated the differentiation-inducing potential of antineoplastic agents.[9-11,13,26] The studies by Marchal et al.[10,13] further revealed that the predominance of differentiation induction or selective destruction may depend on the chemotherapeutic agent and/or dosage. Taken together, the earlier and current findings suggest heterogeneity in rhabdomyosarcomas with respect to both chemosensitivity and differentiation level. It is likely that the better-differentiated cells are more chemoresistant, which appears to be conveyed by expression of LRP (Figure 3).

The clinical relevance of the aforementioned findings remains to be established. Controversy exists with respect to the significance of tumor cell differentiation in relation to survival.[5,7] No clear distinction has been made between residual tumors after chemotherapy and (late) relapses, which may represent different phenomena. *In vitro* studies have demonstrated both decreased proliferative activity and decreased tumorigenicity after treatment with Ara-C.[35] A small series of tumor specimens obtained before and after chemotherapy found an apparent association between differentiation, decreased proliferative activity, and favorable prognosis.[5] In view of the current findings, (pre)terminal differentiation induced by chemotherapy might increase LRP-related MDR, which should be taken into consideration when planning further treatment.

Figure 3. Relationship between differentiation, chemotherapy, and LRP expression. Small arrows indicate progressive morphologic differentiation from "primitive cells" to immature and mature "round rhabdomyoblasts" and "strap cells". D, desmin; –/+/++ negative / moderately/ strongly positive. Large arrows indicate the presumed increasing or decreasing effects of chemotherapy compared with morphology.



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Chapter 6

Multidrug resistance proteins in primary and metastatic soft tissue sarcomas: downregulation of P-glycoprotein during metastatic progression.

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Abstract

Chemotherapy sensitivity of soft-tissue sarcomas (STS) is limited, which may be due to multidrug resistance (MDR). MDR is associated with expression of P-glycoprotein (P-gp), Multidrug Resistance-associated Protein 1 (MRP1) and Lung Resistance-related Protein (LRP). It is unknown whether in STS metastasis is more resistant than the primary counterpart.

In 35 chemonaive STS and their metastases (86% chemonaive), MDR proteins were immunohistochemically assessed. Eleven metastases presented synchronously, 24 metachronously. Expression was scored positive (>5% positive tumor cells) or negative.

P-gp was positive in 31/34 primaries (91%), versus 22/32 metastases (69%) (P=0.005). This difference was significant for metachronous metastases (P=0.008). MRP1 was positive in 18/32 primaries (56%), and 22/33 metastases (67%). MRP1 was more expressed in synchronous metastases than primaries (P=0.047), but for the overall group this significance disappeared. LRP expression did not differ: 27/34 primaries (80%), versus 28/34 metastases (82%).

P-gp, MRP1, LRP expression in the primary tumors was high. Metastatic progression did not coincide with MDR-protein upregulation.

Introduction

Soft-tissue sarcomas (STS) are a heterogeneous group of mesenchymal tumors. Many histological types have been described, which often show differences in biological propensities and clinical behavior. Even within a single histological type, subgroups with different biological make-up and clinical outcome can be distinguished. Taken together, almost half of the STS patients develop metastases in due course. Most of the metastases present as pulmonary lesions; lymph node metastases have been described in ca 3% of STS.[1] The highest incidence of lymph node metastases has been described for angiosarcomas, embryonal rhabdomyosarcomas and epithelioid sarcomas.[1,2] Bone metastases are found in 7% -10 % of STS [3], predominantly in rhabdomyosarcomas. Once metastasized, cure is difficult to achieve. In case a limited number of metastases are present, metastasectomy can be applied with curative intent. However, for the majority of patients with metastasized STS, chemotherapy will be the only option. The relative resistance to chemotherapy of STS in adulthood is at

least partly due to multidrug resistance. This nowadays well-know phenomenon covers resistance to a variety of cytotoxic drugs, such as anthracyclines, vinca-alkaloids and epipodophyllotoxins. Expression of P-glycoprotein (P-gp), the multidrug resistance protein 1 (MRP1) and lung resistance protein (LRP) plays a role in this multidrug resistance. In adult STS, doxorubicin and ifosfamide are the most effective drugs.

There are a few reports studying the expression of these MDR proteins in primary tumors and metastases, which might give an indication about the correlation of P-gp, MRP1 and LRP and metastatic potential.[4-7]

Because of the clinical problem of drug resistance in STS and the high expression of the multidrug resistance proteins P-gp, MRP1 and LRP in STS [8], we wondered whether there might be even an upregulation of one or more of these proteins during the process of metastatic progression. We therefore studied paired samples of primary and metastatic STS from 35 patients treated at our hospital.

Materials and methods

Patients with a STS, of which samples of both the primary tumor and metastasis were available, were retrieved from the computerized files of the Department of Pathology at the University Hospital Groningen (the Netherlands).

Sarcomas were reviewed on hematoxylin and eosin stained paraffin embedded sections with additional immunostains and were classified according to Enzinger and Weiss.[9] Metachronous metastases were defined as metastases detected after 3 months of diagnosis of the primary tumor. Synchronous metastases were those detected within 3 months of diagnosis.

Immunohistochemistry. Immunohistochemistry with the indirect peroxidase method was performed as described previously.[10] The most viable parts of the tumor were selected from the available blocks. The following monoclonal antibodies were used: *C494* to P-gp (Signet Laboratories Inc.; dilution 1:200); *MRPr1* to MRP1 (kindly provided by Dr R.J. Scheper, Free University Hospital, Amsterdam, the Netherlands; dilution 1:15); and *LRP clone 42* to LRP (Transduction Laboratories; dilution 1:400). As positive controls for P-gp, MRP1 and LRP, liver, lung and colon were used, respectively. Immunohistochemistry was scored by two independent observers, having no knowledge of the clinical data. The

scoring was performed semi-quantitatively as positive (>5% immunoreactive tumor cells) or negative.

Statistics. Changes in the expression of MDR- proteins were assessed by analyzing the paired samples of primary tumors and their corresponding metastases by a Wilcoxon signed rank test. For statistical analysis, Statistical Package for the Social Sciences (SPSS) 10.0 for Windows (SPSS Incorporated, Chicago IL, USA) was used.

Results

Between 1981 and 1997 paired samples of 35 STS could be retrieved. In 11 cases metastases had occurred synchronously and in 24 cases metachronously. The following histological types of STS were diagnosed: 3 liposarcomas, 3 angiosarcomas, 3 epithelioid sarcomas, 4 synovial sarcomas, 4 rhabdomyosarcomas, 4 leiomyosarcomas, 5 malignant fibrous histiocytomas (MFH), 5 sarcomas not otherwise specified (NOS) and 4 other sarcomas (1 malignant schwannoma, 1 malignant hemangio-pericytoma, 1 fibrosarcoma, 1 clear cell sarcoma). The localization of the metastases was lung in 12 (34%), lymph node in 11 (31%), soft tissue in 7 (20%), bone in 2 (6%), pleural in 2 (6%) and mesenterial in 1 (3%). Lymph node metastases were found in 2 cases of RMS, 1 epithelioid sarcoma, 1 clear cell sarcoma, 3 MFH, 1 LMS, and 2 NOS. Whereas all samples from primary STS were chemotherapy naïve, this was the case in 30 (86%) of the metastases; 5 (14%) patients had received chemotherapy, after which the metastasis was removed.

Expression of P-gp, MRP1 and LRP is summarized in Table 1. P-gp was expressed in 31/34 primary tumors (91%), versus 22/32 metastases (69%). Paired analysis (primary vs. metastasis) revealed significantly less P-gp positive samples in the metastases group ($p=0.005$). This difference seems to be due to the metachronous metastases group in which far less P-gp positivity was found in the metastases than in the primaries ($p=0.008$). In the synchronous metastases group, no significant difference in P-gp expression was found between primaries and metastases.

MRP1 was positive in 18/32 primary tumors (56%) and in 22/33 metastases (67%). LRP was positive in 27/34 primary tumors (80%), versus 28/34 metastases (82%).

MRP1 and LRP expression did not significantly differ in the total group of primary tumors versus their cognate metastases. However, subgroup

Table 1. Semi-quantitative assessment of P-gp, MRP1 and LRP in primary and corresponding metastatic soft tissue sarcoma.

		P-gp		MRP1		LRP	
Primaries	positive	31	*91%*	18	*56%*	27	*80%*
	negative	3	*9%*	14	*44%*	7	*20%*
	missing	1		3		1	
Metastases	positive	22	*69%*	22	*67%*	28	*82%*
	negative	10	*31%*	11	*33%*	6	*18%*
	missing	3		2		1	

Wilcoxon signed ranks test shows that P-gp expression is significantly different: p=0.005. Both MRP1 and LRP expression are not significantly different between primary versus metastasis.

analysis revealed that in the group with synchronous metastases MRP1 was significantly more often positive in metastases than in primary STS (p=0.046). This was not the case for the metachronous metastases group.

After subgroup analysis for the synchronous and metachronous metastases groups, still no difference between metastases and primaries was found for LRP. Subgroup analysis of the chemotherapy-pretreated metastases could not be performed, due to the limited sample size.

It was found that expression of each of the three MDR associated proteins did not differ for lymph node metastases versus the group of non-lymph node metastases. P-gp, MRP1 and LRP expression was the same for the group of lymph node metastases versus their paired primaries, as for non-lymph node metastases versus their primary counterparts.

Discussion

Despite the substantial burden of literature about multidrug resistance, still several questions remain unanswered and results of studies are at times conflicting. Many papers deal with the physiological and tumor biological role of P-glycoprotein. The role of efflux pumps extruding xenobiotics from normal cells to protect them from harm and the barrier role, e.g. in the blood brain and blood testis barrier are well known.[11,12] In solid tumors, P-gp expression is a major obstacle to the effectiveness of a broad variety of natural cytotoxic agents. In STS, P-gp expression (or MDR1 gene expression, encoding for P-gp) has been related to the response to chemotherapy in childhood and adult STS.[13-20] In addition, the role of P-gp

in the course of metastatic progression has been postulated, but has not yet been clarified.[21,22]

In this study, P-gp was expressed in a high percentage of tumor samples. In a previous study of our group we found a somewhat lower P-gp expression in primary STS (79% of the cases were P-gp positive).[8] The difference between these studies might well have been caused by the selection of metastasizing tumors and the greater share of histological types known for extensive P-gp expression in the present study, like synovial sarcomas, rhabdomyosarcomas and MFH. The finding of the significantly lower number of P-gp expressing metastases than their matching primaries was rather unexpected. The significance of this result seems to be related to the metachronous metastases, i.e. metastases detected beyond 3 months after diagnosis of the primary tumor. Previously, Mattern did not find a difference in P-gp expression in lung tumors in paired primary and simultaneously resected lymph node metastases.[4] In a comparable sample size as the present study P-gp was not upregulated in post-chemotherapy resected metastases from melanomas, when compared with the matched chemonaive primary melanomas.[6] In epithelial ovarian cancer also MDR1 expression did not differ significantly between primary and metastatic sites.[23] In a murine osteosarcoma tumor model MDR1 gene expression was studied during tumor progression. No significantly different levels of MDR1 gene expression were found between primary, recurrent and metastatic lesions.[24] Finally, Zochbauer-Muller observed also a slightly lower expression of P-gp in lymph nodes of breast cancer patients versus the paired primaries.[7]

MRP1 function is increasingly explored.[25] Apart from resistance to the classical MDR drugs, MRP1 can also cause resistance to methotrexate. The clinical significance of high expression of MRP1 in solid human tumors remains to be elucidated. Limited data are available on the role of MRP1 in STS. Oda et al found a relation between the expression of MRP1- RNA and the malignancy grade of STS.[26] Previously, MRP1 expression was found in 67/136 primary STS (49%) [8], which is an only slightly lower percentage than the 56% MRP1 positive samples presented here. The former study consisted of a relatively higher share of leiomyosarcomas and myxoid liposarcomas, which types turned out to be MRP1 negative in a substantial amount of cases.[8]

Although a subgroup analysis showed an increase in MRP1 for synchronous metastases (which was not observed for metachronous metastases), this was of borderline statistical significance. Of interest,

Zochbauer-Muller observed in breast cancer patients elevated MRP1 expression in lymph node metastases compared to primary tumors.[7]

LRP has been identified as the major vault protein (MVP), the main component of multimeric vault particles. The exact role of LRP is still under investigation, although causality between LRP and drug resistance has been demonstrated.[27,28] More recently in MVP (LRP) knockout mice the sensitivity of their bone marrow and stem cells to cytotoxic agents was studied. Based on these experiments their conclusion was that MVP/vaults had no direct role in resistance to chemotherapeutics.[29] Analogous to P-gp and MRP, LRP expression has been studied as potential predictor of response to chemotherapy.[30]. Whether LRP is upregulated during the metastatic process of a tumor has not been investigated before. Previously we demonstrated in 141 primary STS, LRP expression in 74% [8], which is in line with the positivity of the present study including metastasized STS only. From the results of the current study we conclude that LRP expression remains stable during the metastatic process in STS.

There are still some other issues to address for the interpretation of our results. In the present study the number of lymph node metastases is relatively high. The reason for this was merely clinical: lymph node metastases are removed relatively easily for both diagnosis and treatment. Radiographically overt pulmonary lesions in a patient with a high grade STS are highly suspect of metastatic progression. Rarely, such pulmonary lesions are removed with curative aim. This explains why the number of lung metastases in the present study is disproportionate to the clinical situation. Therefore, whether the findings of the current study are representative for the clinical situation remains to be shown in a larger cohort of STS patients with lung metastases.

Related to the high incidence of lymph node metastases in our study is the fact that certain histological types, such as angiosarcoma and rhabdomyosarcoma, were relatively over-represented. This does not reflect the common distribution of histological subtypes in STS.[31]

The question whether expression of multidrug resistance proteins in metastases increases after exposure to chemotherapy can only be answered in studies with adequate sample size. Given the fact that histological types of STS display different biological behavior, this should ideally be done per single type. In a study dealing with rhabdomyosarcomas, chemotherapy appeared to upregulate LRP expression.[32] In this particular study, the majority of post-chemotherapy samples were taken from residual primary lesions; only two metastases were available, both cases showing increased LRP expression compared to the chemotherapy naive primary.

To obtain material of a substantial number of pulmonary metastases, i.e. the commonest site of metastasis, is hampered by ethical constraints. In this respect, it is a pity that European Organization for Research and Treatment of Cancer (EORTC) study STBSG 62933, a spin-off from the study by van Geel [33] and randomizing between preoperative chemotherapy versus direct surgical removal of lung metastases, had to be stopped prematurely because of too slow patient accrual.

In conclusion, metastases of STS do not show a higher expression of multidrug resistance proteins P-gp, MRP1 and LRP than their primary counterparts. On the contrary: P-gp expression was lower in the group of metachronous metastases than in their primary tumors. This is in line with the observation that, also in case of metastatic disease of STS, doxorubicin still induces responses in 20-30% of the patients.

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Chapter 7

Expression of P-glycoprotein, multidrug resistance-associated protein 1, and lung resistance-related protein in human soft tissue sarcomas before and after hyperthermic isolated limb perfusion with tumor necrosis factor-α and melphalan

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Abstract

Multidrug resistance (MDR) is associated with expression of P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and lung resistance-related protein (LRP). Tumor necrosis factor-α (TNF-α) is able to modify the expression of these three proteins in different cell types. The effect of TNF-α in the clinical situation on patients with soft tissue sarcomas (STS) is indeterminate.

Thirty-seven patients with a locally advanced extremity STS underwent hyperthermic isolated limb perfusion (HILP) with TNF-α and melphalan; 15 patients received additional interferon-γ. Clinical and histologic responses were documented and used to define the overall response. Samples before and after HILP were analyzed immunohistochemically for P-gp, MRP1, and LRP. Samples were scored as negative or positive (< 5% or ≥ 5% positive tumor cells).

Six patients had an overall complete response, 25 patients had a partial response, and 4 patients with STS revealed no change; in 2 patients the response remained unclear. The percentage STS samples that were positive for all three proteins dropped from 92% before HILP to 85% after HILP. P-gp positive samples were encountered more often than MRP1 positive samples ($P < 0.05$). The percentage of samples that were negative for all three MDR proteins increased after HILP from 6% to 16%. MDR status had no significant correlation with tumor response.

HILP with TNF-α and melphalan results in excellent overall tumor response in patients with locally advanced STS. STS more often are positive for P-gp than for MRP1. MDR status in patients with STS is not predictive for tumor response after HILP. Data from the current study suggest that the combination of TNF-α and melphalan does not induce MDR positive STS: a result with clinical importance when consecutive, adjuvant, doxorubicin-containing chemotherapy is considered.

Introduction

Soft tissue sarcomas (STS) comprise a rare and diverse group of malignancies of mesenchymal origin. STS tend to metastasize early, mainly hematogenously. High histologic tumor grade of an STS is the major adverse prognostic marker for the development of distant recurrence; 40% of patients with Grade 3 tumors develop metastases.[1-3] The role of

adjuvant chemotherapy remains controversial with respect to the development of distant metastases. However, recent reports indicate a trend toward a beneficial effect in a selected group of patients: those with tumors arising in the extremities.[4-6]

At the Groningen University Hospital, patients with primarily irresectable extremity STS are treated with TNF-α and melphalan in a setting of hyperthermic isolated limb perfusion (HILP). This regimen was a major breakthrough as a limb-salvaging technique. It renders the tumor resectable and avoids amputation in more than 75% of patients.[7,8] Interferon-γ (IFN-γ), initially combined with TNF-α, had no additional benefit on tumor response but increased toxicity and was removed from the schedule.[9] Local tumor control may be improved further by using adjuvant external beam radiotherapy.[10]

Like with other tumors, the phenomenon of multidrug resistance (MDR), in which tumors are resistant to various structurally and functionally different natural product chemotherapeutic drugs, also is encountered in STS.[11-14] MDR is associated with the overexpression of P-gp, MRP1, and LRP.[13,15] Both P-gp and MRP1 act as transmembrane pumps, excreting xenotoxins out of cells toward the extracellular environment; P-gp also prevents toxins from crossing the cell membrane.[16] A recent study revealed a correlation between P-gp expression in high grade STS and poor outcome to chemotherapy consisting of doxorubicin, dacarbazine, and ifosfamide.[17] LRP, which was discovered in a lung carcinoma cell line, is described as the major vault protein.[18,19] The exact function of LRP is not understood, and its causal relation to MDR has been established only recently.[20] LRP vaults are present in the cytoplasm and in the nuclear membrane and may mediate transport of (cytotoxic) substrates from the nucleus to the cytoplasm.[21,22] This leads to an altered drug distribution within the cell, resulting in lower amounts of the cytotoxic agent at the nuclear target site.[20] LRP is involved in resistance to classical drug resistance-associated agents (doxorubicin, etoposide, and paclitaxel) and also to melphalan.[21] In multiple myeloma, its expression is identified as an independent predictor for resistance against the alkylating drug melphalan, although, in multiple myeloma, this resistance can be overcome by dose intensification.[23]

The cytokine TNF-α enhances the effect of antineoplastic agents in various ways, from affecting the tumor-associated vasculature, direct cytotoxicity, enhancement of proliferation (rendering the tumor more susceptible to melphalan), and increasing the concentration of cytotoxic agents (e.g., melphalan) in the tumor interstitium.[24-31] TNF-α also has the

ability to reduce drug resistance by modulation of the expression of drug resistance-associated proteins.[32,33] TNF-α decreases the expression of P-glycoprotein (P-gp) and lung resistance-related protein (LRP) but increases multidrug resistance-associated protein 1 (MRP1) expression in colon carcinoma cells *in vitro*.[32,33] After exposure to TNF-α, these cells were more susceptible for classic multidrug resistance (MDR)-related agents, like doxorubicin and vincristine.[32] P-gp also protects drug-resistant cells from caspase dependent apoptosis, which is the pathway for cell death induced by the TNF-death receptor family.[34]

The first objective of the current study was to assess the expression of MDR proteins in patients with STS before and after TNF-α exposure (with or without IFN-γ). Second, the correlation between tumor response to therapy and the expression of P-gp, MRP1, and LRP were be investigated.

Materials and Methods

Patients. Thirty-seven patients with a primarily irresectable extremity STS consented to undergo HILP. Four tumors were located in the upper extremity, and 33 tumors were located in the lower extremity. In 35 of 37 patients, a delayed marginal resection of the tumor remnant was performed 6–8 weeks after HILP (61 days ± 16 days; range, 12–103 days; median, 60 days); no complementary therapeutic interventions were planned during the period between HILP and the resection. The study was approved by the Medical Ethical Committee of the University Hospital Groningen. Paraffin embedded tumor samples were collected from the time of diagnosis and from the time of resection after HILP.

Histologic diagnosis. In all patients, the diagnosis was made on hematoxylin and eosin-stained paraffin sections from incisional biopsies, sometimes with additional immunohistologic staining. Sarcomas were classified according to Enzinger and Weiss [35], revealing 16 different histologic types (Table 1). Tumor grading was performed according to the grading system developed by Coindre et al.[36] This resulted in 7 patients with Grade 1 STS, 19 patients with Grade 2 STS, and 11 patients with Grade 3 STS (Table 1). The extent of necrosis in the resection specimen was estimated on macroscopic examination. For histology, at least one section per centimeter of the greatest tumor dimension was taken. The presence of necrosis, viable tumor, or fibrosis was documented histologically.

Table 1. Patient characteristics

Patient	Sex	Age (Yrs)	Histological type	Grade	IFNγ	Clinical response	Histologic response	Overall response
1	M	18	Extraskeletal myxoid chondrosarcoma	2	+	PR	PR	PR
2	F	18	Rhabdomyosarcoma	2	+	PR	CR	CR
3	M	21	Liposarcoma	2	–	PR	PR	PR
4	M	22	Epitheloid sarcoma	2	+	PR	NC	NC
5	M	24	Synovial sarcoma	2	–	CR	PR	PR
6	F	25	Synovial sarcoma	3	–	PR	PR	PR
7	F	26	Sarcoma NOS	3	–	PR	Unk	Unk
8	M	28	Sarcoma NOS	3	+	CR	NC	PR
9	F	33	Leiomyosarcoma	2	–	PR	CR	CR
10	F	37	MPNST	2	–	PR	PR	PR
11	M	37	Myxoid liposarcoma	1	–	PR	PR	PR
12	F	39	Synovial sarcoma	3	–	CR	PR	PR
13	F	40	Leiomyosarcoma	3	+	PR	PR	PR
14	M	42	Clear cell sarcoma	1	–	PR	PR	PR
15	M	43	Synovial sarcoma	2	+	NC	NC	NC
16	M	44	Myxoid liposarcoma	1	–	PR	PR	PR
17	F	44	Myxoid liposarcoma	1	+	PR	PR	PR
18	F	47	Myxoid MFH	1	+	PR	CR	CR
19	F	48	Well differentiated liposarcoma	1	+	NC	NC	NC
20	M	48	Myxoid liposarcoma	2	–	NC	PR	PR
21	M	49	Malignant hemangiopericytoma	1	–	PR	PR	PR
22	F	50	Dedifferentiated liposarcoma	3	+	PR	PR	PR
23	F	50	Sarcoma NOS	2	–	CR	CR	CR
24	M	53	MFH	2	+	PR	PR	PR
25	F	53	Sarcoma NOS	2	–	PR	CR	CR
26	M	54	MFH	3	–	Unk	Unk	Unk
27	F	56	PPNET	2	–	PR	CR	CR
28	F	60	Leiomyosarcoma	3	+	PR	PR	PR
29	F	61	Myxoid MFH	2	+	PR	PR	PR
30	M	62	MPNST	2	+	NC	NC	NC
31	F	64	MPNST	2	–	PR	PR	PR
32	M	66	MFH	2	–	PR	PR	PR
33	F	67	MFH	3	–	PR	NC	PR
34	F	69	Fibrosarcoma	3	–	PR	PR	PR
35	M	71	Liposarcoma	2	+	NC	PR	PR
36	M	74	Sarcoma NOS	3	–	PR	PR	PR
37	M	80	Sarcoma NOS	2	–	PR	NC	PR

M, male; F, female; NOS, not otherwise specified; MPNST, malignant peripheral nerve sheath tumor; MFH, malignant fibrous histiocytoma; PPNET, peripheral primitive neuroectodermal tumor; CR, complete response; PR, partial response; NC, no change; Unk, unknown.
Histologic classification according to Enzinger and Weiss.[35]
Grading according to Coindre, Trojani et al.[36]
Tumor response assessment according to Eggermont et al.[7,9]

Drugs and treatment schedule. HILP was performed as described previously.[9] Briefly, the major artery and vein were exposed surgically and canulated after systemic heparinization (3.3 mg/kg body weight). Isolation of the limb circulation was achieved by clamping the major artery and vein, by ligation of collateral vessels, and by applying a tourniquet. The circulation of the affected limb was carried out by a heart-lung machine.

Recombinant human TNF-α (Beromun™, Boehringer Ingelheim Pharma GmbH & Co.KG, Ingelheim am Rhein, Germany) was used: 3 mg TNF-α for perfusions of an arm and 4 mg for perfusions of a leg. Melphalan (GlaxoSmithKline, London, Great Britain) was administered in a dosage of 10 mg (leg) or 13 mg (arm) per liter of limb volume. IFN-γ (Boehringer Ingelheim Pharma GmbH & Co.KG) was administered to 15 patients in addition to TNF-α and melphalan. The dosage used was 0.2 mg IFN-γ subcutaneously on the 2 days before HILP and 0.2 mg intra-arterially at the start of HILP.

IFN-γ and TNF-α were injected as a bolus into the arterial line. Melphalan was administered 30 minutes thereafter. The procedure consisted of a 90-minute perfusion (counted from the time of TNF-α injection) at mild hyperthermia (39–40 °C). At the end of the perfusion, the limb was flushed with 500 mL Isodext (NPBI, Emmer-Compascuum, the Netherlands) in NaCl 0.9% with 250 mL red blood cell concentrate. The heparin-induced anticoagulative state was corrected with protamine sulfate.

Immunohistochemical detection of MDR proteins. Samples were deparaffinated in xylene and alcohol. Antigen retrieval was achieved by heating the samples at 115 °C under pressure (10 psi) in an autoclave in three cycles of 5 minutes each. The monoclonal C494 (Signet Laboratories, Dedham MA, USA; 120 mg/mL; dilution, 1:200), which recognizes P-gp, was used. For MRP1, a noncommercial monoclonal antibody was used (MRPr1; kindly provided by Dr. R. J. Scheper, Department of Pathology, Free University Hospital, Amsterdam, the Netherlands; concentration, 20 mg/ mL; dilution, 1:15). Anti-LRP monoclonal antibody (Transduction Laboratories, Los Angeles, CA, USA; 250 mg/ mL) was used at a 1:400 dilution. The samples were incubated with the primary antibody-containing dilution at room temperature for 1 hour. For each sample, a peroxidase-conjugated secondary antibody identified the binding of the primary antibody. Diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO, USA) in phosphate-buffered saline was used as the chromagen. Samples were counterstained with hematoxylin. Liver, lung, and colon tissue served as positive controls for P-gp, MRP1, and LRP expression, respectively. The expression of P-gp, MRP1 and LRP was scored by estimating the

percentage of positive stained tumor cells. All samples were scored at the same time by the same investigators (B.E.C.P. and H.H.) without knowledge of the response to the therapy. The score was documented as the percentage of positive staining tumor cells. In concordance with former publications, samples were classified as positive for MDR expression if < 5% of the tumor cells showed immunoreactivity.[15,37,38]

Assessment of tumor response. Tumor response after HILP with TNF-α (with or without IFN-γ) and melphalan was assessed according to the standard scoring system developed by Eggermont and coworkers.[9] Overall tumor response was derived from both clinical and histologic responses.

The greatest tumor dimension was used as the parameter for clinical response, as assessed by physical examination and radiodiagnostic imaging. A clinical complete response (CR) was defined as the disappearance of all measurable disease for > 4 weeks. A partial response (PR) was classified as regression of the tumor by > 50% for > 4 weeks. No change (NC) was concluded when regression of < 50% or progression of < 25% of the tumor existed for > 4 weeks. Clinical progressive disease (PD) was defined as > 25% disease progression. Responses were assessed by standardized World Health Organization criteria.[39]

Extensive histopathologic examination of the resected specimens was performed. The percentage of viable tissue was estimated on the basis of macroscopic and histologic examination. Histologic CR was defined as 100% microscopic disappearance of viable tumor tissue, histologic PR was defined as > 50% viable tumor tissue, and histologic NC was defined as < 50% viable tumor tissue.

If no tumor was detectable clinically (*clinical* CR), then the final outcome was downgraded to a PR when the resection specimen showed viable tumor tissue. If the clinical response was a PR, then the final response could upgrade to a CR only if histologic examination of the tumor remnant showed 100% necrosis or fibrosis. Likewise, if treatment resulted in clinical regression of < 50% but the tumor was resectable, and histologic examination showed that the tumor remnant was ≥ 50% necrotic, then the overall response was upgraded to a PR.

Statistical analysis. Alterations in the expression of MDR proteins in pre- HILP and post-HILP samples were assessed by using the Pitman test with correction for continuity. The McNemar test was used to analyze differences between scoring of the distinct MDR proteins in the same specimen. The Pearson chi-square test was used to determine the correlation between tumor response and expression of MDR proteins.

Results

Thirty-seven patients with an irresectable STS of the limb underwent HILP with TNF-α and melphalan; 15 patients received additional IFN-γ. Two patients provided no sample for histologic examination after HILP: One patient had progressive lung metastases requiring chemotherapy (Patient 7), and the other patient underwent an amputation of the affected limb 2 days after HILP due to vascular occlusion (Patient 26).

Tumor response to HILP with TNF-α and melphalan. A clinical CR was observed in four patients (11%). Twenty-seven patients (73%) revealed a clinical PR, whereas, in 5 patients (14%) a clinical NC was detected.

A histologic CR was detected in 6 patients (16%), and a histologic PR was detected in 22 patients (60%). In 7 patients (19%) the tumor showed a histologic NC. Histologic responses remained undetermined in two patients (5%).

From the clinical and histologic responses, the overall tumor response was constructed. Six patients (16%) had a CR, and 25 patients (68%) had a PR. In four patients (11%) the overall tumor response was designated NC. In two patients, the overall response was unknown. Table 1 provides an overview of these results.

MDR proteins in patients with STS before and after HILP with TNF-α and melphalan.

Pre-HILP samples. One sample for P-gp and LRP (from the same specimen) and three samples for MRP1 were not taken into account due to uncertainty about the presence of representative tumor material. The results of the scoring are shown in Table 2.

A positive score for at least one MDR protein was established in 34 of 37 specimens (92%). Samples from the same specimen were positive for P-gp more frequently than for MRP1 (25 vs. 15 samples, respectively; $P < 0.05$). Co-expression of P-gp and MRP1 was found in 13 of 33 samples (39%), co-expression of P-gp and LRP in was found 17 of 36 samples (47%), and co-expression of MRP1 and LRP was found in 12 of 33 samples (36%). Positive scoring for all three proteins was found in 7 of 33 samples (21%). Samples from three patients were scored negative for all three proteins.

Post-HILP samples. Of the 35 remaining histologically evaluable STS samples, 6 revealed pathologic CR to the treatment, leaving no viable tissue for immunohistochemistry. In addition, samples were not evaluable for P-gp, MRP1, or LRP in 2 patients, 3 patients, and 4 patients,

respectively, because of a lack of representative tumor material. The results of the scoring are shown in Table 3.

Table 2. MDR protein expression *before* HILP

	P-gp		MRP1		LRP	
	No.	%	No.	%	No.	%
Negative	9	(25%)	18	(53%)	13	(36%)
Positive	27	(75%)	16	(47%)	23	(64%)
Missing	1		3		1	

Table 3. MDR protein expression *after* HILP

	P-gp		MRP1		LRP	
	No.	%	No.	%	No.	%
Negative	6	(22%)	16	(62%)	10	(40%)
Positive	21	(78%)	10	(38%)	15	(60%)
Missing	10		11		12	

Scoring of P-gp, MRP1 and LRP expression in patients with soft tissue sarcomas *before* (Table 2), and *after* (Table 3) hyperthermic isolated limb perfusion with tumor necrosis factor-α and melphalan.

The number of samples that scored positive for at least one MDR protein decreased to 23 of 27 samples (85%) compared with 34 of 37 samples (92%) before HILP. Samples derived from the same STS more often were scored positive for P-gp than for MRP1 (20 vs. 10 samples, respectively; $P < 0.05$). Both P-gp and MRP1 staining were positive in 10 of 26 samples (38%); and both P-gp and LRP staining were positive in 13 of 25 samples (52%). Co-expression of MRP1 and LRP was present in 9 of 25 samples (36%). Positive scoring for all three proteins was present in 9 of 25 STS samples (36%). A negative score for all three proteins was encountered in 4 of 25 samples (16%).

Changes in immunohistochemical staining of MDR proteins before and after HILP. The scoring for P-gp, MRP1 and LRP separately did not

significantly alter for paired samples from the same tumor before and after HILP with TNF-α and melphalan (with or without IFN-γ). Stratification for the use of IFN-γ did not alter these results. Figure 1 displays the results of the scoring of the three MDR proteins before and after HILP.

Of the three patients with STS samples that were negative for all three MDR proteins prior to HILP, two patients had positive MDR samples after HILP, whereas the third patient remained negative for all three. After undergoing HILP, a higher percentage of patients with P-gp negative, MRP1 negative, and LRP negative samples was found: 16% (4 of 25 samples) compared with 6% (3 of 33 samples) before HILP, but this did not reach a level of statistical significance. In Table 4, the absolute percentages of positive P-gp, MRP1, and LRP results before and after HILP are presented. Within the positively scored samples (> 5% positivity), there is a wide variation in the absolute percentage of antibody staining. However, no uniform trend in either up-regulation or down-regulation can be discerned.

MDR Expression Related to Tumor Response. The expression of P-gp, MRP1, and LRP in STS prior to chemotherapy was not correlated with clinical, histologic, or overall tumor response. No significant differences in the expression of MDR were found between the subgroups of patients with distinct tumor responses (CR, PR, and NC). The number of patients in the current series was too small to draw conclusions concerning differences in MDR expression among different histologic entities.

All three patients with MDR negative samples before HILP had an overall PR. In the category of patients with no or low tumor response (NC) to the therapy, there were no significant differences in scoring of MDR proteins compared with the good responders (PR). Samples that indicated a histologic CR could not be taken into account.

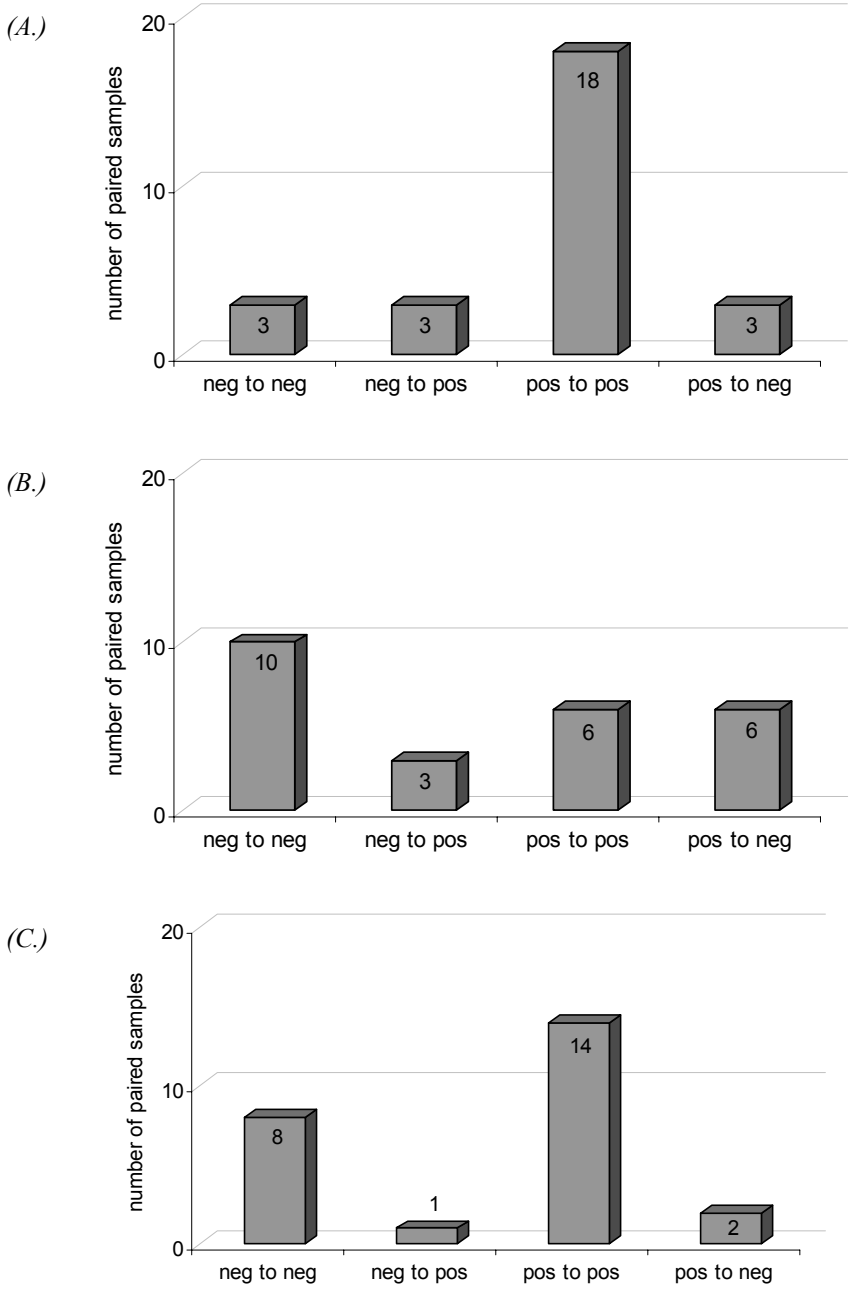

Figure 1. Immunohistochemistry for P-gp *(A.)*, MRP1 *(B.)*, and LRP *(C.)* in soft tissue sarcomas before and after HILP with TNF-α and melphalan

Table 4. Results of immunohistochemistry: absolute percentages of tumor cells stained positively before and after HILP with TNF-α and melphalan.

Patient	Histologic type	P-gp		MRP1		LRP	
		Before	After	Before	After	Before	After
1	Extraskeletal myxoid chondrosarcoma	40	40	5	NE	70	NE
2	Rhabdomyosarcoma	5	CR	0	CR	70	CR
3	Liposarcoma	80	20	40	70	90	70
4	Epitheloid sarcoma	20	20	NE	80	40	80
5	Synovial sarcoma	0	90	0	70	0	90
6	Synovial sarcoma	10	30	0	0	90	70
7	Sarcoma NOS	40	NE	0	NE	5	NE
8	Sarcoma NOS	30	60	70	60	90	90
9	Leiomyosarcoma	5	CR	NE	CR	60	CR
10	MPNST	70	40	40	50	50	90
11	Myxoid liposarcoma	80	90	0	0	0	0
12	Synovial sarcoma	90	80	90	20	0	0
13	Leiomyosarcoma	0	0	80	0	30	30
14	Clear cell sarcoma	40	40	40	0	0	0
15	Synovial sarcoma	20	40	30	0	0	0
16	Myxoid liposarcoma	90	20	0	0	0	0
17	Myxoid liposarcoma	5	10	0	0	0	NE
18	Myxoid MFH	NE	CR	90	CR	NE	CR
19	Well differentiated liposarcoma	40	0	10	0	0	0
20	Myxoid liposarcoma	0	0	0	0	5	0
21	Malignant hemangiopericytoma	20	0	0	0	0	0
22	Dedifferentiated liposarcoma	20	NE	20	NE	0	NE
23	Sarcoma NOS	10	CR	90	CR	50	CR
24	MFH	40	0	0	0	60	0
25	Sarcoma NOS	0	CR	0	CR	90	CR
26	MFH	60	NE	NE	NE	70	NE
27	PPNET	90	CR	50	CR	5	CR
28	Leiomyosarcoma	5	0	0	0	60	30
29	Myxoid MFH	90	90	0	90	70	70
30	MPNST	0	20	30	0	20	20
31	MPNST	50	90	10	0	30	90
32	MFH	50	NE	0	NE	70	NE
33	MFH	80	80	0	0	90	0
34	Fibrosarcoma	50	70	0	90	80	80
35	Liposarcoma	40	80	1	1	90	80
36	Sarcoma NOS	30	50	70	70	70	90
37	Sarcoma NOS	50	60	40	90	90	90

NE, not evaluable; CR, complete histologic response; NOS, not otherwise specified; MPNST, malignant peripheral nerve sheath tumor; MFH, malignant fibrous histiocytoma; PPNET, peripheral neuroectodermal tumor.

Discussion

The combined use of biologic and chemotherapeutic agents provides a new approach of dealing with drug-resistant tumors. For patients with STS, the combination of TNF-α and melphalan results in an excellent tumor response, although the mechanisms of action remain to be clarified. The HILP technique allows the use of TNF-α and melphalan in dosages that are intolerable in the systemic circulation. The main aim of HILP is limb salvage. The increase in the limb salvage rate due to HILP with TNF-α and melphalan is not reflected by an increase in the distant failure rate.[8,9]

Sarcomas are known to exhibit a drug-resistant phenotype, even when they are unexposed to chemotherapy.[11,13] Therefore, it has been suggested that MDR status may be a determinant for tumor response to therapy, even though TNF-α and melphalan are not the classic substrates for P-gp, MRP1, and LRP. However, it has been demonstrated *in vitro* that P-gp can protect tumor cells against TNF-α-induced apoptosis.[34] Furthermore, LRP may play a role in melphalan resistance.[23] The lack of correlation found in the current study between the expression of MDR proteins and tumor response implies that MDR does not play a major role when STS are treated with high doses of TNF-α and melphalan under hyperthermic conditions.

In the current study, chemotherapy-naive STS were positive for at least one MDR protein in 97% of patients; after TNF-α and melphalan perfusion, this number decreased to 85%. However, it must be noted that the complete responders could not be taken into account.

MDR modification by cytokines has been investigated extensively in the *in vitro* situation; the current survey permits insight into the actual clinical situation. This study showed that the combination of TNF-α and melphalan in a setting of HILP did not lead to the selection of clones with high expression of P-gp, LRP, and/or MRP1 in STS. This is supported by the increasing numbers of P-gp negative, MRP1 negative, and LRP negative specimens after HILP. No pattern of change in the three MDR proteins could be detected. One problem in the interpretation of these results is the timing of the second sample.[40] The timing of residual tumor resection in the current study was based on clinical judgment when the tumor was alleged to be resectable.[9] Considering the period after which MDR protein expression may be expected, the time span between TNF-α exposure and second sampling is rather long (61 ± 16 days). For comparison, in colon carcinoma cells, the decline of MDR1 gene

expression (encoding for P-gp) already starts within 48 hours after exposure to TNF-α.[41] *In vivo* studies confirm a fast mechanism in the modification of drug resistance: activation of MDR1/P-gp expression in pulmonary sarcoma metastases occurs within 1 hour after doxorubicin exposure.[42] Although the identification of P-gp in the study by Abolhoda et al.[42] was on mRNA level, a subsequent increase of the functional protein may be expected.[43] In this view, it is noteworthy that TNF-α is able to down-regulate MDR proteins, such as P-gp and LRP, whereas doxorubicin quickly up-regulates MDR1/P-gp.[32,33,42] What happens to the MDR-status in the group with histologic CR could not be studied. To elucidate this issue, samples should be taken earlier after HILP. Then, when viable tissue still exists, one has a view into the important early mechanisms in MDR modulation. However, this approach is accompanied by practical and ethical problems, because early sampling implements exposure of the patient to additional invasive procedures.

Using HILP, leakage into the systemic circulation cannot always be prevented.[44,45] Because STS are known for early hematogeneous metastasizing, potentially present synchronous micrometastases are exposed to the same drugs as the target tumor when leakage occurs. In addition, although metastatic cells per se do not have the biologic behavior of the cells from the primary tumor, it is reassuring that HILP with TNF-α and melphalan did not increase further the MDR status of the primary tumor. This is an important issue, because most adjuvant chemotherapy schemes are based on typical MDR-related anticancer agents.

In conclusion, HILP with TNF-α and melphalan results in good clinical and histologic tumor response in patients with STS. Expression of the MDR proteins P-gp, MRP1, and LRP in chemotherapy-naive patients with STS is not predictive for tumor response. The numbers of MRP1 positive STS results were significantly lower compared with the numbers of P-gp positive STS results, both in the pre-HILP samples and the post-HILP samples. In the patients who achieved either PR or NC, tumor response has no correlation with changes in MDR status before and after HILP with TNF-α and melphalan; the group of patients who achieved a CR could not be evaluated. The current study suggests that HILP with TNF-α and melphalan does not lead to a selection of MDR positive tumors: a result with possible clinical implications when adjuvant doxorubicin-containing chemotherapy is applied.

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Chapter 8

Effects of TRAIL, doxorubicin and 4-hydroxy-ifosfamide in a panel of soft-tissue sarcoma cell lines with different sensitivity to tumor necrosis factor-family cytokines

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Abstract

Doxorubicin (DOX) and ifosfamide (IFO) are the most active single agents in soft tissue sarcomas (STS). Still, the response rate in metastatic disease is only 20-30%. Tumor necrosis factor-α (TNF-α) is used for STS only in the setting of isolated limb perfusions. Like TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) also induces apoptosis, and preliminary studies indicate that TRAIL lacks systemic side effects. Resistance to TRAIL has been demonstrated, but can be circumvented by combinations of TRAIL with conventional cytotoxic agents, *in vitro*. The effects of TRAIL alone and in combination with DOX or 4-hydroxy-IFO (i.e. the active metabolite of IFO, 4-OH-IFO) were evaluated in a panel of TNF-α sensitive and resistant human soft tissue sarcoma cells.

The rhabdomyosarcoma cell line KYM-1, its five-fold TNF-α sensitive subline KD4 and its >150-fold TNF-α resistant subline 37B8R were used. Membrane expression of the TRAIL-receptors DR4, DR5 (pro-apoptotic) and DcR1, DcR2 (anti-apoptotic) was assessed by flow cytometry. Drug-induced cytotoxicity was determined by a microculture tetrazolium assay. Apoptosis assays using acridine orange were conducted for the combination that was most potent of inducing cytotoxicity.

DOX and 4-OH-IFO decreased survival in all cell lines; a two-fold resistance was observed for both drugs in 37B8R. All three cell lines expressed DR4 and DR5, but no or very low levels of DcR1 or DcR2. TRAIL single agent decreased survival in KYM-1 and was even more cytotoxic in KD4 and induced massive apoptosis, while 37B8R was >500-fold resistant to TRAIL compared to KYM-1 and little apoptosis could be observed. The combination of TRAIL plus DOX showed synergistic cytotoxic effects in KYM-1 and 37B8R. The combination of TRAIL plus 4-OH-IFO showed to be additive in all three cell lines. DOX plus TRAIL induced more cytotoxicity as well as apoptosis in all three cell lines compared to TRAIL alone. In 37B8R, DOX overcame resistance to TRAIL.

In KYM-1 and its sublines KD4 and 37B8R, sensitivity and resistance to TNF-α and TRAIL parallels. TRAIL resistance was independent from expression of TRAIL receptors. DOX with TRAIL could overcome TRAIL-resistance in 37B8R cells, suggesting a therapeutic potential for this combination for TNF-α and TRAIL refractory STS cells.

Introduction

Soft tissue sarcomas (STS) are the group of malignancies of mesenchymal origin. In case of metastatic disease, curation is difficult to reach with standard treatment, including doxorubicin (DOX) and ifosfamide (IFO). Therefore, finding alternative agents is critical.

A potential innovative treatment implies the use of cytokines of the tumor necrosis factor (TNF) superfamily. Some members of this growing family have drawn attention as anticancer agents, including the prototype TNF-α that rapidly induces apoptosis in many cancer types. TNF-α in combination with melphalan is used in the setting of a hyperthermic isolated limb perfusion, resulting in a local response rate up to almost 90% in locally advanced STS.[1] However, this regional treatment has no impact on the metastasis-free and overall survival of patients, while the systemic use of TNF-α is hampered by severe toxic side-effects.[2,3]

TNF-related apoptosis-inducing ligand (TRAIL) is a more recently identified member of the TNF superfamily that selectively induces apoptosis in malignant cells.[4,5] Native TRAIL is safe in non-human primates, while human tissues are spared at tumoricidal concentrations, suggesting that TRAIL is a candidate for systemic cancer treatment.[6-8] TRAIL is a type II transmembrane protein and so far, four membrane-bound receptors for TRAIL have been described: DR4, DR5, DcR1 and DcR2. Apoptosis is mediated by the two death receptors DR4 and DR5, while the two decoy receptors DcR1 and DcR2 interrupt apoptosis.[9]

The combination of TRAIL with cytotoxic agents offers several possibilities. First, TRAIL can increase sensitivity to cytotoxic agents, or even restore sensitivity in resistant cells. Second, cytotoxic agents can increase sensitivity to TRAIL-mediated apoptosis. Third, when combinations proof to be more effective than the single agents, lower amounts of the cytotoxic agents and TRAIL can be applied.

The aim of the present study was to analyze the effect of cytotoxic agents (DOX and activated IFO) with the new apoptosis-inducing agent TRAIL in a panel of isogenic soft-tissue sarcoma cell lines. The panel consisted of the rhabdomyosarcoma cell line KYM-1, its TNF-α sensitive subline KD4 and its TNF-α resistant subline 37B8R. Cytotoxicity of the cytotoxic agents and TRAIL was tested alone and in combination. In addition, TRAIL receptor status of the cell lines was evaluated and apoptosis studies were performed under conditions of the most effective cytotoxic combinations.

Materials and Methods

Chemicals. RPMI 1640 medium, L-glutamine and sodium pyruvate were obtained from Invitrogen (Merelbeke, Belgium), fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands). TNF-α was kindly provided by Boehringer-Ingelheim (Ingelheim am Rhein, Germany). Recombinant human soluble TRAIL was made according Ashkenazi et al.[6] and was dissolved in medium with 10% FCS at a stock concentration of 423 µg/ml. The active metabolite of IFO, 4-hydroxy-IFO (4-OH-IFO) was a gift from Asta Medica (Frankfurt, Germany). DOX (Adriablastina™) was obtained from Pharmacia & Upjohn (Woerden, The Netherlands). Sodium azide and acridine orange (AO) were purchased from Sigma (St Louis, MO, USA); AO was dissolved in demineralized water at a 1-mg/ml concentration. The ready-to-use tetrazolium dye solution Cell Proliferation Reagent WST-1 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anti-DR4, -DR5, -DcR1, -DcR2 antibodies were a gift from Amgen (formerly Immunex; Thousand Oaks, CA, USA). FITC-labelled rabbit-anti-mouse antibodies were bought from DAKO (Glostrup, Denmark).

Cell lines and culturing. KYM-1, an embryonal rhabdomyosarcoma cell line [10] and the two sublines KD4 and 37B8R were kindly provided by A. Meager (National Institute for Biological Standards and Control, Potters Bar, UK). KYM-1 has been described as a TNF-α sensitive cell line.[11-17] KD4 was established after limited dilution cloning of KYM-1 cells and has increased sensitivity to TNF-α.[11,18,19] 37B8R cells are resistant to TNF-α, established after exposure to gradually increasing concentrations of TNF-α.[11] The cell lines were grown in RPMI 1640 medium supplemented with 7% FCS, 1mM L-glutamine and 1mM sodium-pyruvate. Cells were incubated at 37°C with saturated humidity and 5% CO_2. All three cell lines grow in suspension under increasing medium viscosity due to the production of hyaluronic acid.[10] To avoid reproducibility problems due to handling cells under viscous conditions, cells were harvested for experiments at latest two days after addition of fresh medium.[19] New passages of all three cell lines were started every three months.

Cell viability assays. For cytotoxicity measurements, a modified microculture tetrazolium assay was used. This colorimetric assay is based on cleavage of the tetrazolium dye WST-1 (4-[3-(4-iodophenyl)-2-(4-

nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to water-soluble formazan by mitochondrial dehydrogenases in viable cells. The linear association between cell concentrations to the production of the formazan was confirmed and cell growth studies were performed to assure exponential cell growth at the time of measuring cell survival. For KYM-1 and KD4, 1.2×10^4 cells per well and for 37B8R, 1.0×10^4 cells per well were incubated with single agent or combinations of DOX, 4-OH-IFO and TRAIL in a total volume of 200 µl, all in triplo. After 96 hours, 20 µl of the WST-1 solution was added and incubated for 3 hours at 37 °C. Formazan production was assessed by measuring the absorbance at a wavelength of 450 nm on a scanning microplate spectrophotometer (Benchmark™ Microplate Reader, Bio-Rad Laboratories).

Background absorbance was corrected by subtracting the absorbance measured for the culture medium in absence of cells. The percentage of cell survival was calculated as:

% survival = (absorbance of experimental well / absorbance of untreated well) × 100

Each experiment was performed in triplicate, with a minimum of three individual experiments per cell line per drug or combinations of drugs.

Apoptosis assays. AO staining was used to distinguish apoptotic cells from vital cells.[20] To analyze apoptosis induction, 6.0×10^4 cells per well were incubated in triplo with different concentrations of cytotoxic agent and/or TRAIL in a total volume of 200 µl into a 96-wells plate. Untreated cells were used as controls. After 24 hours of exposure, AO (final concentration: 5 ng/ml) was added and incubated at 37 °C for 10 minutes. Then cells were centrifuged at 900 rpm for 15 minutes. Three-quarter of the supernatant was removed and checked first for unintentional aspiration of cells before proceeding to counting cells under a fluorescent microscope (Olympus IM, Japan; wavelength 525 nm). The apoptotic index was calculated as:

% apoptosis = (number of apoptotic cells) / (total number of cells)

A minimum of three independent experiments per single agent and combination per cell line was performed.

Analysis of cell viability and apoptosis assays. To analyze the potential enhancing effects of the cytotoxic agents on TRAIL-mediated cytotoxicity

and apoptosis, the enhancement ratio (ER) for each experiment was calculated. ER was calculated by dividing the effect of TRAIL alone by the effect of TRAIL combined with the cytotoxic agent, the latter corrected for the effect of cytotoxic agent alone.[21] In concordance with previous studies, an ER-value between 0.8-1.2 was considered as indicative for additivity, ER lower than 0.8 as antagonistic and ER higher than 1.2 as synergistic.

Expression of TRAIL receptors. Cells (1.0×10^5) were analyzed for DR4, DR5, DcR1 and DcR2 expression by flow cytometry, using receptor NH_2-terminal-specific monoclonal antibodies. First, harvested cell were washed in phosphate buffered saline (PBS) with 2% FCS and 0.1% sodium azide. Cells were then incubated with the primary antibodies at 37 °C for 30 minutes. After washing in PBS with 2% FCS and 0.1% sodium azide, cells were incubated with FITC-labelled rabbit-anti-mouse antibody for 30 minutes while kept on ice. As control for auto-fluorescence, cells that were not incubated with the primary and secondary antibody were analyzed. As control for a-specific binding of the FITC-labelled secondary antibody, cells that were not incubated with the primary antibody were analyzed. Data from KD4 and 37B8R cells were compared with those of the parental KYM-1 cells. Three individual experiments were performed.

Results

Effect of DOX, 4-OH-IFO and TRAIL on cell survival. The sensitivity profiles of the three cell lines to TNF-α as previously described [11] were confirmed by a pilot study (data not shown).

Table 1 shows the sensitivity of the three cell lines to the tested drugs: DOX, 4-OH-IFO and TRAIL. The IC50 (concentration inhibiting cell survival by 50%) level for KYM-1 and KD4 was reached at similar concentrations of both DOX and 4-OH-IFO, while 37B8R was a factor 2 resistant compared to KYM-1 for both drugs.

TRAIL by itself had a strong cytotoxic effect on both KYM-1 and KD4. At the highest concentrations tested (5μg/ml), the cytotoxic effect of TRAIL on KYM-1 was 60%, while for KD4 over 90% cytotoxicity was reached. On the contrary, TRAIL alone had limited effect on 37B8R, never capable of inducing more than 25% cytotoxicity. The drug concentrations used in the combination experiments were titrated on a range of 25 to 75% cytotoxicity (when feasible).

Table 1. IC50 values for single-agents DOX, 4-OH-IFO and TRAIL in KYM-1, KD4 and 37B8R cell lines

	KYM-1		KD4		37B8R	
	mean	*SD*	mean	*SD*	mean	*SD*
DOX [nM]	3.10	± *0.63*	3.84	± *0.45*	6.1	± *2.20*
4-OH-IFO [nM]	254	± *126*	172	± *150*	538	± *261*
TRAIL [µg/ml]	0.0065	± *0.0018*	0.0047	± *0.0037*	>5	

Abbreviations: IC50, concentration inhibiting cell survival by 50%; DOX, doxorubicin; 4-OH-IFO, 4-hydroxy-ifosfamide; TRAIL, tumor necrosis related apoptosis-inducing ligand; SD, standard deviation

Figure 1 shows the effects of combining DOX with TRAIL in the KYM-1 cell line. Both DOX and TRAIL individually decreased cell survival. At all tested concentrations, DOX and TRAIL were able to induce extra cell death. The enhanced cytotoxic effect became also evident in TRAIL-resistant 37B8R cells (Figure 2). For the tested combinations of DOX, 37B8R cells were sensitized to TRAIL-mediated cytotoxicity. When comparing absolute cell survival at isomolar concentrations, KYM-1 was found to be more sensitive to this combination than 37B8R at DOX [3nM] and TRAIL [0.001 µg/ml], but these cell lines were equally sensitive at DOX [15nM] and TRAIL [0.001 µg/ml].

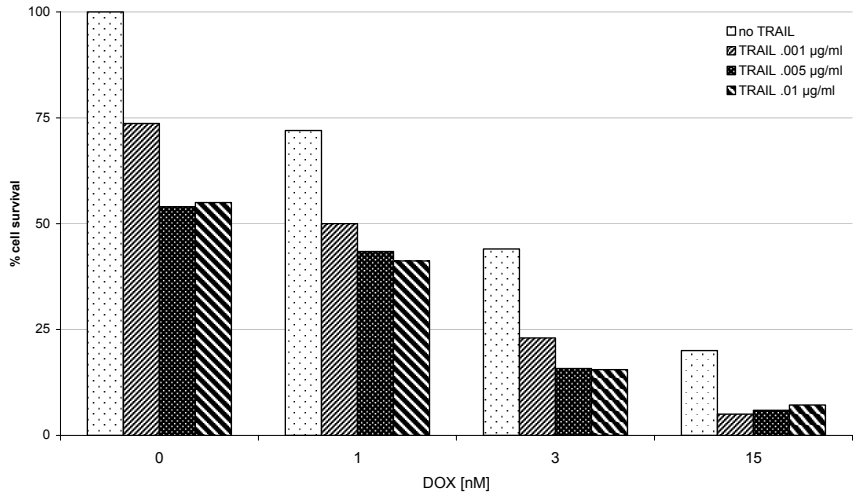

Figure 1. Sensitivity of KYM-1 to DOX, TRAIL and combinations of these two drugs (96-hours continuous incubation) as measured in a modified microculture tetrazolium assay

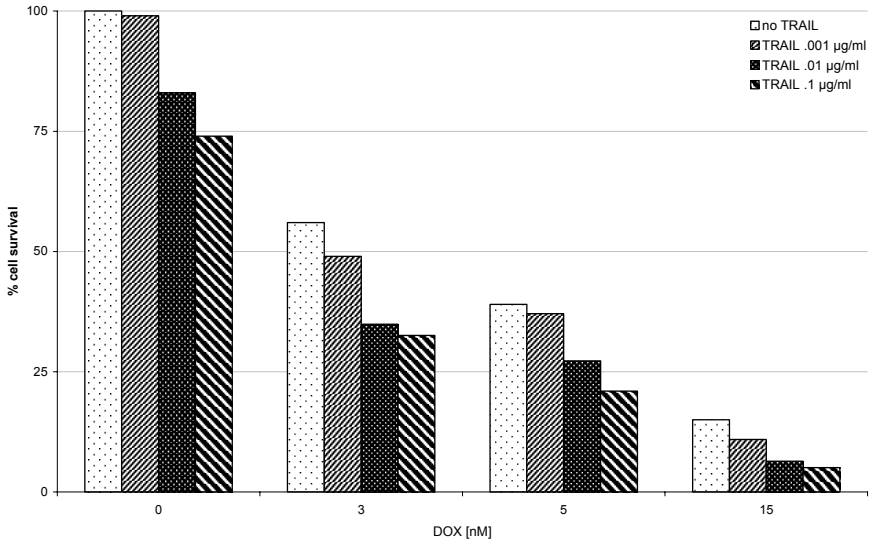

Figure 2. Sensitivity of 37B8R to DOX, TRAIL and combinations of these two drugs (96-hours continuous incubation) as measured in a modified microculture tetrazolium assay

Table 2 summarizes the enhancement ratio (ER) found for the combination of DOX and TRAIL in the three cell lines. Combining TRAIL with DOX shows synergy (ER > 1.20) in especially KYM-1 and 37B8R, with the effect being most stable in the 37B8R cell line.

The combined effects of 4-OH-IFO and TRAIL have been depicted in Table 3, showing the calculated ER's. For all combinations and all cell lines, additivity was observed.

Table 2. DOX and TRAIL

KYM-1		DOX [nM] 1	3	15
	0.001	*1.14 (0.40)*	***1.76*** *(1.07)*	***3.69*** *(1.85)*
TRAIL [µg/ml]	0.005	*0.91 (0.14)*	***1.61*** *(0.44)*	***2.94*** *(2.10)*
	0.01	*0.97 (0.12)*	***1.64*** *(0.42)*	***2.25*** *(1.57)*

Table 3. 4-OH-IFO and TRAIL

KYM-1		4-OH-IFO [nM] 20	80	200
	0.001	*1.02 (0.14)*	*1.15 (0.36)*	*1.09 (0.21)*
TRAIL [µg/ml]	0.005	*0.95 (0.08)*	*0.88 (0.15)*	*0.91 (0.20)*
	0.01	*0.99 (0.21)*	*0.97 (0.26)*	*1.12 (0.32)*

(Table 2. continued)

KD4		DOX [nM] 2	5	25
	.0005	*0.93 (0.24)*	*0.96 (0.24)*	*0.96 (0.24)*
TRAIL [µg/ml]	0.001	*0.98 (0.32)*	*0.98 (0.32)*	***1.53** (0.51)*
	0.005	***1.26** (0.27)*	***1.26** (0.27)*	***2.49** (1.86)*

37B8R		DOX [nM] 3	5	15
	0.001	*1.14 (0.07)*	*1.06 (0.11)*	***1.41** (0.15)*
TRAIL [µg/ml]	0.01	***1.34** (0.05)*	***1.21** (0.10)*	***2.06** (0.25)*
	0.1	***1.33** (0.20)*	***1.54** (0.46)*	***2.77** (1.52)*

(Table 3. continued)

KD4		4-OH-IFO [nM] 20	200	500
	.0005	*1.09 (0.10)*	*0.95 (0.13)*	*1.01 (0.17)*
TRAIL [µg/ml]	0.001	*1.12 (0.22)*	*1.07 (0.39)*	***1.37** (0.50)*
	0.005	*0.89 (0.06)*	*0.99 (0.25)*	***1.43** (0.57)*

37B8R		4-OH-IFO [nM] 20	200	500
	0.001	*1.01 (0.15)*	*0.96 (0.03)*	*1.07 (0.07)*
TRAIL [µg/ml]	0.01	*0.96 (0.16)*	*0.95 (0.06)*	*0.97 (0.07)*
	0.1	*1.19 (0.23)*	***1.22** (0.27)*	***1.37** (0.32)*

Enhancement ratio's of modified microculture tetrazolium assays for KYM-1, KD4 and 37B8R cell lines at indicated concentrations of DOX and TRAIL (Table 2) and 4-OH-IFO and TRAIL (Table3).
(standard deviation indicated between brackets; synergistic interactions highlighted in bold)

Induction of apoptosis. The combination DOX plus TRAIL was further investigated in apoptosis assays, as this combination gave rise to the most pronounced effects in the cytotoxicity assays. AO staining was used to distinguish apoptotic cells from viable cells.[20] After incubation with AO, apoptotic cells were recognized as cells with condensed chromatin, whether intact or already fragmented, intensely staining with AO. Membrane blebbing with shedding of apoptotic bodies was considered as an alternative hallmark of apoptosis. Viable cells had loosely packed ("extended") chromatin with an intact cellular membrane. Because of the shorter incubation period for apoptosis assays, higher concentrations of TRAIL and DOX were used compared to the modified microculture tetrazolium assays.

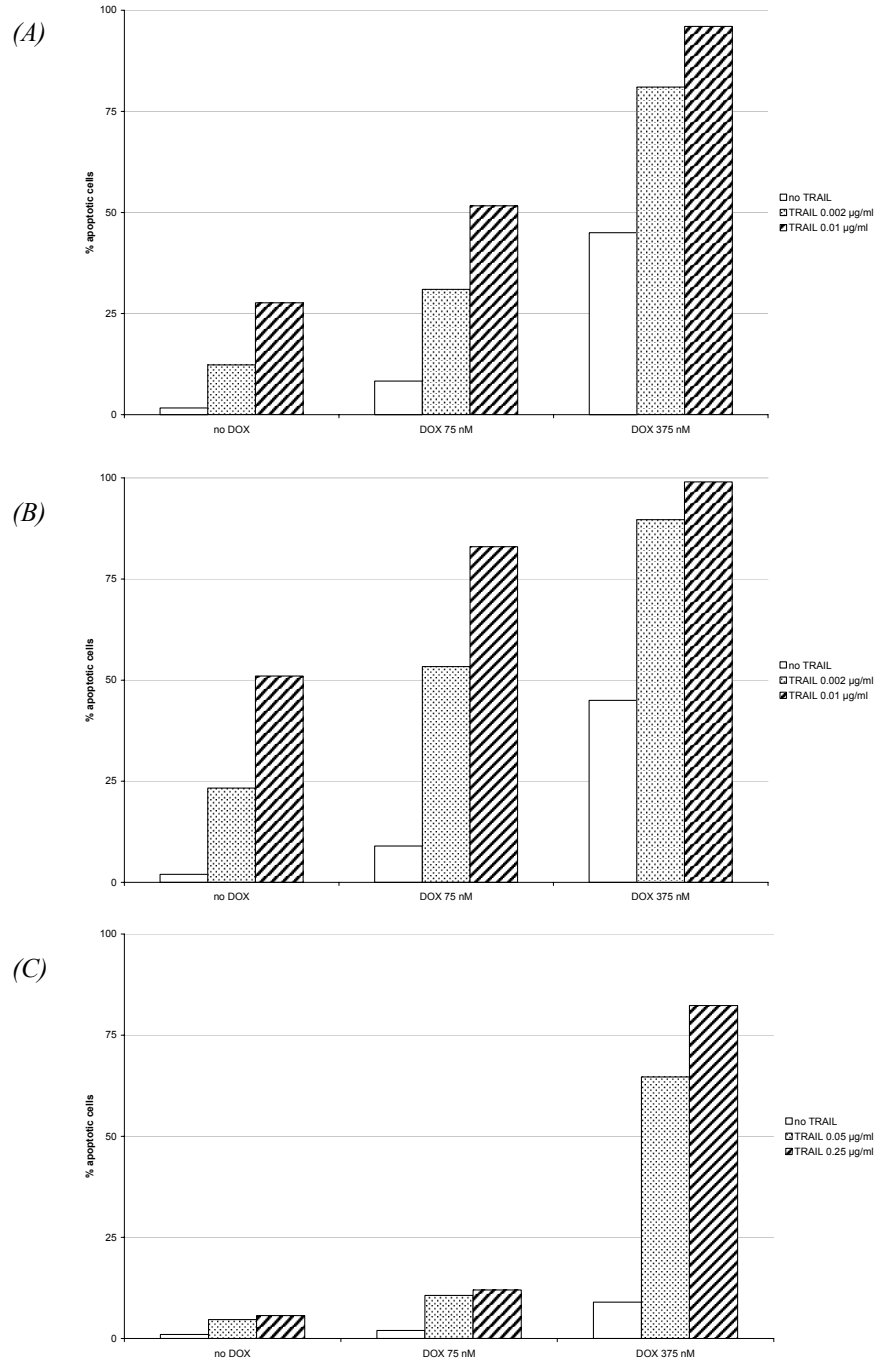

Figure 3. Induction of apoptosis by DOX, TRAIL and combinations of these two drugs (24 h exposure): *(A)* KYM-1; *(B)* KD4; *(C)* 37B8R

Figures 3a, 3b and 3c show the results of apoptosis assays in KYM-1, KD4 and 37B8R, respectively. While single-agent induced apoptosis in KYM-1 and KD4 cells, the combination of the two agents increased the percentage of apoptotic cells. KYM-1 and KD4 revealed similar pattern in sensitivity to TRAIL and DOX; still, apoptosis was more pronounced in KD4. After 24 hours of exposure, more KD4 cells than KYM-1 cells were in an advanced stage of apoptosis, with fragmented chromatin and membrane blebbing. Contrary, a relatively small percentage of 37B8R cells revealed features of apoptosis after exposure to DOX and higher doses of TRAIL (Figure 3c). Still, the combination of TRAIL with DOX resulted in a marked increase of apoptosis (Figure 3c). A dramatic increase of apoptosis in 37B8R was observed when DOX [375 nM] was combined with TRAIL, either [0.05μg/ml] or [0.25μg/ml].

Similar to the analysis of modified microculture tetrazolium assays, ER's were calculated for the induction of apoptosis by the combination of DOX and TRAIL and are show in Table 4. Synergistic apoptosis induction by DOX plus TRAIL was observed for all three cell lines at most concentrations tested. In KYM-1, synergistic ER-values were observed especially at the higher DOX concentrations. Noticeable, the apoptosis induction was quite variable at the highest concentrations. This variability was even higher in KD4 cells, with extremely high ER's as a result of maximal apoptosis induction. Still, at the lower concentrations of DOX [75nM], the effect remains synergistic even though to a lesser degree, yet more stable as indicated by the narrower standard deviation. The synergistic effect of DOX and TRAIL on apoptosis induction was present as well in 37B8R, although less pronounced than in the other two cell lines. However, while TRAIL and DOX alone did scarcely induce apoptosis, their combination showed apoptosis induction comparable to the KYM-1 cell line. Moreover, the synergistic effect in 37B8R at the higher concentrations of DOX [375 nM] was found to be the most stable of all three cell lines. Higher concentrations of TRAIL were required for 37B8R to obtain similar levels of apoptosis compared to KYM-1, illustrative of a more resistant phenotype.

Table 4. Enhancement ratio's of apoptosis induction in KYM-1, KD4 and 37B8R cell lines at indicated concentrations of DOX and TRAIL.
(standard deviation indicated between brackets; synergistic interactions highlighted in bold; *n.t.* = not tested)

KYM-1		DOX [nM]		
		15	75	375
TRAIL [µg/ml]	0.002	*n.t.*	*1.18 (0.07)*	***2.60** (0.40)*
	0.01	***1.22** (0.14)*	***1.42** (0.26)*	***7.14** (4.15)*
	0.05	***1.69** (0.71)*	***2.85** (0.90)*	*n.t.*

KD4		DOX [nM]		
		15	75	375
TRAIL [µg/ml]	0.002	*n.t.*	***1.54** (0.21)*	***18.4** (18.7)*
	0.01	*1.05 (0.32)*	***3.16** (0.88)*	***28.8** (11.8)*
	0.05	*0.81 (0.38)*	***2.59** (2.86)*	*n.t.*

37B8R		DOX [nM]		
		15	75	375
TRAIL [µg/ml]	0.01	*1.01 (0.05)*	*1.06 (0.06)*	*n.t.*
	0.05	*1.08 (0.07)*	*1.06 (0.03)*	***2.55** (0.46)*
	0.25	*n.t.*	*1.06 (0.02)*	***5.60** (2.77)*

TRAIL receptor membrane expression. KYM-1, KD4 and 37B8R expressed DR4 and DR5, while no or very low levels of DcR1 or DcR2 could be detected. Compared to KYM-1, KD4 expressed factor 1.5 to 2 less DR4, and comparable levels of DR5. Compared to KYM-1, 37B8R showed similar expression of both DR4 and DR5.

Discussion

STS comprise a heterogeneous group of malignancies, sharing their origin from primitive mesenchymal cells. As a whole, STS are notorious for early hematogenous spread. The presence of (micro-) metastases limits the curative use of local treatments (surgery and radiotherapy), rendering chemotherapy as the only tumor-directed therapy.

Anticancer drugs exert their cytotoxic effect through the induction of apoptosis in tumor cells. Blockades in the complex routes leading to apoptosis may consequently convey resistance to anticancer drugs. One means to circumvent the resistance to cytotoxic agents is combining them

with alternative apoptosis inducing agents. TRAIL is a newly described apoptosis-inducing member of the TNF superfamily of cytokines, which might be of value for these purposes.[22]

The aim of the present study was to analyze the combined effect of the two most active cytotoxic agents in STS, i.e. DOX and activated IFO, with the new apoptosis-inducing agent TRAIL in a panel of rhabdomyosarcoma cell lines.

The cell lines selected for the current study were initially reported for their sensitivity to TNF-α, the prototype of the TNF superfamily. In later studies, additional data became available for the sensitivity profile to other TNF family cytokines: TRAIL, Fas Ligand, TWEAK.[23,24] This panel represents a unique model of related soft tissue sarcoma cell lines with different sensitivity to the TNF family of cytokines, including TRAIL. These characteristics allow exploring the effects of combining cytotoxic agents with TRAIL on TRAIL-sensitive, but even more importantly, TRAIL-resistant STS cells.

DOX and 4-OH-IFO were both capable of inducing cytotoxicity in all three cell lines. As the cell lines have been established in the absence of cytotoxic anticancer agents, no mechanisms of drug resistance driven by the exposure to such drugs could have been evolved. Still, the IC50 to DOX and 4-OH-IFO for the TNF-α/TRAIL resistant cell line 37B8R was higher than for the TNF-α/TRAIL sensitive cell lines KYM-1 and KD4. KYM-1 and KD4 were both sensitive to TRAIL-mediated cytotoxicity. However, while for KD4 nearly complete cell kill could be achieved, a survival of approximately 40% of KYM-1 cells was still observed at the highest tested concentration of TRAIL (5 μg/ml). Caron et al. reported that KD4 cells were resistant to TRAIL at concentrations up to 0.2 μg/ml.[19] This difference with the current study might be attributed to molecular differences in TRAIL used (we used native TRAIL versus His-tag TRAIL in the study of Caron) and the method to detect apoptosis (morphological versus flow cytometric analysis of annexin V flip-flop to the external membrane leaflet). The concentrations of TRAIL applied in the present study are corresponding to those of former *in vitro* and *in vivo* studies, at which levels clearly identifiable anticancer results were achieved.[6,22] We observed that KD4 was the most sensitive cell line to TRAIL: after 24 hr exposure to TRAIL, KD4 cells were already in an advanced stage of apoptosis. Contrary, TRAIL was able to induce 20% cytotoxicity maximum in 37B8R at the tested range of concentrations. Concerning this TRAIL resistance of 37B8R cells, the current study comes to similar

findings to that of the study by Caron et al. In an earlier study on single-agent TRAIL, Petak et al. reported that four out of a panel of seven rhabdomyosarcoma (not KYM-1) cell lines were sensitive to TRAIL.[25] However, in the three TRAIL-resistant cell lines, an additional factor would have been required to achieve significant anticancer effects. Therefore, in the current study we tested TRAIL single agent, but we also tested the interaction of TRAIL with cytotoxic agents.

Combining DOX with TRAIL resulted in an increased effect on cell kill in all three rhabdomyosarcoma cell lines tested. In KYM-1 and KD4, in which substantial cell kill could be achieved with TRAIL single agent, DOX could still contribute to the cytotoxicity. Interesting, in 37B8R, TRAIL alone lacked a significant cytotoxic effect, whereas combination with DOX was able to overcome TRAIL-resistance. In 37B8R, the synergistic effect as determined by ER was the most stable in repeated experiments.

In the laboratory setting, the combination of DOX with TRAIL has proven to be effective in killing epithelial cancer cell lines: prostate [26-28], breast [29-31], liver [32], lung [30,33], ovarian [30,34], colon [30,35], squamous cell [30], melanoma [30]; hematological cancer cell lines [36-38], and mesenchymal cancer cell lines.[39,40] Clayer et al. described the effects of TRAIL and DOX in primary cultures obtained from three STS of different histological type (rhabdomyosarcoma, fibrosarcoma and malignant fibrous histiocytoma).[41] In their study, TRAIL alone had no cytotoxic effect. The combination of TRAIL, however, led to overtly more cytotoxicity in the respective cell lines than DOX alone. The combined effect of TRAIL and DOX was more pronounced compared to the combinations of TRAIL with cisplatin, etoposide, methotrexate or cyclophosphamide.

To our knowledge, this is the first report on the effect of activated IFO and TRAIL. Overall, combinations showed an additive cytotoxic effect on the three tested cell lines. Evdokiou reported on TRAIL with cyclophosphamide, a drug that is structurally related to IFO, on osteogenic sarcoma cells.[40] In these cells, cyclophosphamide alone did not induce cytotoxicity within the tested range of concentrations, nor did it enhance TRAIL-mediated cytotoxicity. However, it remains uncertain whether the investigators had used cyclophosphamide as a pro-drug or in its activated form. Of interest, drug resistant tumor cells can develop cross-resistance to pro-apoptotic cytokines.[42] As mentioned, combining these cytokines with cytotoxic agents might still overcome this resistance. In the current model the combination of DOX and TRAIL prevailed over that of 4-OH-IFO and

TRAIL. The molecular mechanisms by which DOX and TRAIL aid each other in killing cancer cells remain to be investigated.

The underlying mechanism of sensitivity to TRAIL in tumor cells is poorly understood. Apoptosis is mediated by the two death receptors DR4 and DR5, while the two decoy receptors DcR1 and DcR2 interrupt apoptosis. The cell lines presented here expressed similar levels of DR4 and DR5, while DcR1 and DcR2 were absent or nearly detectable. While expression of the death receptors DR4 and DR5 is essential for TRAIL-induced apoptosis, the presented results suggest that their level of expression is not determinative for the sensitivity to TRAIL. Several factors are uncovered that can influence this machinery to reach this final goal. Amongst these are the pro-apoptotic factors caspases 8 and 10 [9], and Bax [43], and the anti-apoptotic factor FLIP [44], the family of inhibitors of apoptosis proteins (IAP's) [45] and the Bcl-2 family.[46] Much is yet unclear about the contribution of these individual factors and it appears conceivable that differences exist on their role between tumors. Laboratory studies continue to uncover the very multifactorial nature of resistance to TRAIL. In this context it is difficult to define a clinically applicable target to circumvent TRAIL-resistance. Meanwhile, as suggested by the current study and others, conventional cytotoxic agents appear a realistic approach to this purpose. Still, it remains a challenge to search for non-toxic agents with these properties.

In the rhabdomyosarcoma cell line KYM-1 and its sublines KD4 and 37B8R, sensitivity to TNF-α and TRAIL paralleled. TRAIL-sensitivity and -resistance was independent from TRAIL receptor expression, suggesting that one or more downstream apoptotic blocks exist in TRAIL resistant 37B8R cells. The data presented here show that in the TRAIL sensitive lines KYM-1 and KD4, enhanced cytotoxicity could be achieved when combining TRAIL with DOX or 4-OH-IFO. Importantly, in the TRAIL resistant cell line 37B8R, DOX with TRAIL could overcome TRAIL-resistance, suggesting a therapeutic potential for this combination for TNF-α and TRAIL refractory STS cells.

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Chapter 9

Clinicopathologic assessment of postradiation sarcomas: KIT as a potential treatment target

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Abstract

Postradiation sarcoma, a sarcoma developing in a previously irradiated field, is a rare tumor. Surgery appears to be the only curative treatment option. In general the prognosis is poor, and new treatments options are needed. One study reported the expression of KIT receptor tyrosine kinase in two postradiation angiosarcomas. Success of inhibition of KIT in malignant gastrointestinal stromal tumors with imatinib mesylate seems mutation-dependent, with a favorable response in the presence of exon 11 mutations.

We performed a clinical, immunohistochemical, and genetic assessment of postradiation sarcomas, including angiosarcomas. Archival tumor tissue was available from 16 patients diagnosed with a postradiation sarcoma between 1978 and 2001. Data on the first and secondary tumor, treatment, and follow-up was documented. KIT expression was assessed by immunohistochemistry. For comparison, 23 spontaneous soft tissue sarcomas of similar histological types were analyzed. Exon 11 of the *c-kit* gene was analyzed by direct DNA sequencing.

Fifteen patients received initial irradiation for malignant disease and 1 patient for a benign condition. The median delivered dose was 50 Gy. The median latency period between irradiation and diagnosis of postradiation sarcomas was 222 months. Histological types included: angiosarcoma, fibrosarcoma, malignant fibrous histiocytoma, osteosarcoma, rhabdomyosarcoma, and unspecified sarcoma. In concordance with the literature, patients had a poor outcome. Only 3 of 16 patients were disease-free 43, 60, and 161 months after being diagnosed of postradiation sarcoma, all 3 having favorable tumor and treatment characteristics. Fourteen of 16 tumor samples were KIT-positive (88%). In 8 cases >80% of tumor cells stained positively. Five of 23 (22%) spontaneous soft tissue sarcomas of comparable histological types, including 2 angiosarcomas, were KIT-positive. Molecular genetic analysis of exon 11 of the *c-kit* gene was attainable for 13 of the 16 postradiation sarcomas. No mutations were found.

Postradiation sarcomas are aggressive malignancies, seldom amenable to curative treatment. A majority of the analyzed tumors showed extensive expression of the KIT protein, but no mutations in exon 11 of the *c-kit* gene were found. Still, without the availability of effective therapies, treatment with the KIT inhibitor imatinib mesylate might be considered for patients with postradiation sarcomas.

Introduction

Sarcomas developing in previously irradiated fields are rare. Nevertheless, these so-called postradiation sarcomas pose a major clinical problem. In general, surgery is the only curative treatment; still, even radical surgery does not prevent recurrence of the disease in a majority of cases. The role of additional radiation therapy is limited, because the maximum tolerated cumulative dose to the target region has often already been reached. Moreover, in case radiotherapy can be applied, postradiation sarcomas appear to be radioresistant. The role of chemotherapy in the treatment of postradiation sarcomas is also very limited.[1]

The report of Miettinen *et al.*[2] including two postradiation angiosarcomas expressing KIT (c-kit protein) prompted us to study a larger series of postradiation sarcomas. The *c-kit* gene is the cellular homologue of the *v-kit* oncogene of the Hardy-Zuckerman 4 feline sarcoma virus [3] and is located on the long arm of chromosome 4. It encodes the KIT transmembrane receptor tyrosine kinase, which is involved in cell signal transduction. KIT is consistently expressed in malignant gastrointestinal stromal tumors (GISTs) [4], the most common sarcomas of the gastrointestinal tract. KIT appears to play a major role in the oncogenesis of these tumors.[5,6] Mutations of the *c-kit* gene leading to ligand-independent activation of KIT tyrosine kinase are common in malignant GISTs.[5,7] Like postradiation sarcomas, GISTs are notoriously resistant to standard cytotoxic agents.[8] However, imatinib mesylate (Glivec in Europe, Gleevec in the United States; Novartis Pharma) is able to induce apoptosis in GIST cells *in vitro* by inhibiting KIT activity.[9] In the clinical situation, imatinib mesylate has been reported to induce promising clinical and radiological tumor response rates in patients with metastasized GISTs.[10,11] Noticeable, it appears that tumors bearing an activating exon 11 mutation of the *c-kit* gene are the most responsive to imatinib mesylate.[12]

Imatinib mesylate and other KIT-targeted agents may have therapeutic potential for malignancies other than GISTs, which are also subjected to a KIT-mediated oncogenic drive. Given the preliminary data on postradiation angiosarcomas, KIT expression was assessed on a series of 16 postradiation sarcomas with various histological diagnoses.

Materials and Methods

Using the computerized files of the department of Pathology at the University Hospital Groningen, data from 27 patients with a postradiation sarcoma were retrieved. Of these 27 patients, frozen and/or paraffin-embedded tumor material was available in 16 cases. These patients were diagnosed, treated, and/or referred for consultation between 1978 and 2001. The criteria for postradiation sarcoma included: (*a*) different histopathologic features between index lesion (*i.e.,* indication for initial radiotherapy) and sarcoma; (*b*) sarcoma arising within the irradiated field; and (*c*) a latency period of at least 3 years.[13,14] Sarcomas were reviewed on H&E-stained sections with additional immunostains and classified according to Enzinger and Weiss.[15]

Patient demographics, tumor characteristics (of both the index lesion and the postradiation sarcoma), treatment, and follow-up were documented. The latency period was calculated from the moment of initial radiotherapy until the diagnosis of the postradiation sarcoma.

Cytogenetics. Of four postradiation sarcomas, a karyotype was obtained. Fresh tumor material was cultured for 5–15 days in RPMI 1640 (Life Technologies, Inc.), supplemented with 13,5% FCS, L-glutamine, and penicillin/streptomycin. Cultures were harvested, and chromosome samples were made according to standard cytogenetic techniques. The metaphases were air dried and stained with Giemsa after G banding with either trypsin (Difco; Fisher Scientific, Hertogenbosch, the Netherlands) or pancreatin (Sigma, St. Louis, MO).

Immunohistochemistry. For detection of KIT the rabbit polyclonal antibody A-4502 (DAKO, Glostrup, Denmark) was used in a 1:100 dilution. First, samples were deparaffinated in xylene and rehydrated in alcohol. As described by others, heat-induced epitope retrieval was performed to facilitate epitope-antibody interaction.[7,16-18] Samples were heated in 0.1 M Tris-hydrogen chloride (pH 9.0) for 8 min in a microwave (700 watt). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS before proceeding to a 1-h incubation with the primary antibody. Next, a biotin-streptavidin immunoperoxidase method was applied, using biotinylated swine antirabbit IgG (1:300; DAKO) and streptavidin conjugated to horseradish peroxidase (1:300; DAKO). Bound peroxidase was developed with diaminobenzidine and hydrogen peroxide.

Normal small intestine tissue was used as a positive control, demonstrating KIT-positive interstitial cells of Cajal within in the muscular

layers.[19] As internal positive controls, melanocytes (for samples including epithelial layers) and mast cells were to be detected.

Samples were scored as negative when no immunoreactive tumor cells were observed. Positive samples were semiquantitatively categorized according to the percentage of immunoreactive tumor cells: <50%, 50–80%, and >80%. For comparison of the immunohistochemistry, 23 spontaneous soft tissue sarcomas were studied for KIT expression, using an identical immunohistochemical procedure. This control group included histological types similar to the postradiation group. Sarcoma types that are not or only seldom reported in association with prior irradiation (*e.g.,* liposarcoma and synovial sarcoma) were omitted from this study.

Genetic analysis of exon 11 of c-KIT. DNA was isolated from frozen or paraffin-embedded material using standard methods.[20] Sequence analysis of exon 11 of the *c-KIT* gene was performed on PCR products made with the following two M13 tailed primers: cKit-for 5'-CGACGTTGTAAAACGACGGCCAGTTTTGTTCTCTCTCCAGAGTG- 3' and the cKit-rev 5'-CAGGAAACAGCTATGACAGTCACTGTTATGTGTACCC-3'. Direct sequencing using M13 primers in both sense and antisense directions was performed using the BigDye terminator sequencing kit V-3.1 (Applied Biosystems) and an ABI PRISM 377 DNA sequencer (PE Biosystems).

Results

Patient demographics, tumor data, and treatment schedules are summarized in Table 1. The study group consisted of 9 females and 7 males. The median patient age at time of initial irradiation was 29 (range, 2–72) years. Fifteen patients received radiation therapy for a malignant index lesion. As part of clinical routine of the sixties, 1 patient received 16 Gy before diagnostic biopsy of a suspected osteosarcoma. However, the definitive histopathological diagnosis in this case was an ossifying myositis, a nonmalignant condition.

Irradiation dose and additional treatments. The total delivered irradiation dose was known in 12 cases, with a median of 50 Gy and range from 16 to 70 Gy. In 4 cases information on the irradiation dose was not retrievable, all involving a prolonged latency period after radiotherapy (≥32 years). Six patients had received systemic therapy for the index lesion as well; 5 were treated with cytotoxic agents, and 1 patient was treated with hormones.

Table 1. Patient, tumor and treatment characteristics

Patient	Sex	Index lesion				
		Age at diagnosis	Type	Surgery	Chemotherapy	RT (Gy)
1	F	47	Breast ca.	BCT	No	50
2	F	72	Breast ca.	BCT	No	70
3	F	63	Breast ca.	BCT	Hormonal	70
4	M	35	Ossifying myositis	Incision	No	16
5	F	43	Adenoca. uterus	Hysterectomy	No	Unk
6	M	16	m. Hodgkin	No	MOPP-ABV	60
7	F	28	m. Hodgkin	No	MOPP-ABV	40
8	F	22	m. Hodgkin	No	Vinblastine/ chlorambucil	35
9	M	12	RMS maxilla	No	VAC	55
10	M	2	Retinoblastoma	Enucleation	No	45
11	F	21	m. Hodgkin	No	No	40
12	F	18	m. Hodgkin	Unk	Unk	Unk
13	M	25	Osteo	Resection	No	Unk
14	M	68	SCC mouth	Resection	No	60
15	F	30	SCC vulva	Resection	No	Unk
16	M	47	Adenoca. colon	Resection	5-FU/levamisol	50

Patient	Postradiation sarcoma						
	Age at diagnosis	Latency (Months)	Type	Site	Surgery	Chemo	RT (Gy)
1	54	84	Angio	Breast	Mastectomy	No	Yes
2	79	84	Angio	Breast	Mastectomy	No	No
3	67	53	Angio	Breast	Mastectomy	No	No
4	51	204	MFH	Thigh	Resection	VAC/ DTIC	No
5	83	480	MFH	Groin	Resection	No	No
6	22	72	Fibr	Pelvis	No	EC	39
7	34	65	Fibr	Mediastinal	No	VI	No
8	53	372	Osteo	Breast	Resection	No	No
9	33	252	Osteo	Maxilla	Resection	MTX/cisplatin	No
10	22	258	RMS	Nose	No	EVI	No
11	39	240	NOS	Sternal	Resection	EVI	60
12	50	384	NOS	Rib	Resection	Unk	Unk
13	61	432	NOS	Supraorbital	No	No	No
14	71	40	NOS	Mouth	No	No	No
15	71	492	NOS	Vulva	No	No	No
16	55	102	NOS	Pelvis	Resection	No	No

Patient	Follow up				KIT expression
	OS (Months)	LF	DF	Status	% positive cells
1	13	Yes	No	DOD	<50%
2	58	Yes	Yes	AWD	>80%
3	22	No	Yes	AWD	>80%
4	161	No	No	NED	>80%
5	60	No	No	NED	>80%
6	14	Yes	Yes	DOD	50-80%
7	9	Yes	No	DOD	50-80%
8	25	Yes	Yes	DOD	<50%
9	43	No	No	NED	<50%
10	5	Yes	No	DOD	>80%
11	11	Yes	No	DOD	neg
12	Unk	Unk	Unk	Unk	50-80%
13	21	Yes	No	AWD	>80%
14	11	Yes	No	DOD	neg
15	2	Yes	Yes	AWD	>80%
16	2	Yes	No	AWD	>80%

Abbreviations:
SCC, squamous cell carcinoma; Angio, angiosarcoma; MFH, malignant fibrous histiocytoma; Fibr, fibrosarcoma; Osteo, osteosarcoma; RMS, rhabdomyosarcoma; NOS, sarcoma not otherwise specified; BCT, breast conservative treatment; RT, radiotherapy; OS, overall survival; LF, local failure; DF, distant failure; DOD, dead of disease; AWD, alive with disease; NED, no evidence of disease; MOPP/ABV, Mechlorethamin/ vincristine/procarbazine/prednisone/adriamycin/bleomycin/vinblastine; VAC, vincristine/ adriamycin/cyclophosphamide; 5-FU, 5- fluorouracil; DTIC, dacarbazine; EC, epirubicin/ cyclophosphamide; VI, vindesine/ifosfamide; EVI, epirubicin/vindesine/ifosfamide; MTX, methotrexate; Unk, unknown

Latency period. The median latency period between radiotherapy for the index lesion and diagnosis of the postradiation sarcoma was 222 months. The shortest latency period was observed for a patient with a sarcoma not otherwise specified (NOS), 40 months after irradiation with 60 Gy for a squamous cell carcinoma of the floor of the mouth. The longest latency period was seen for a patient who developed a sarcoma NOS 41 years after a squamous cell carcinoma of the vulva (irradiation dose unknown). The median age of patients at time of diagnosis of postradiation sarcoma was 53.3 (range, 22–83) years.

Histological types of postradiation sarcomas. Five different histological types of postradiation sarcomas were diagnosed. Three patients had angiosarcomas, 2 had fibrosarcomas, 2 had osteosarcomas, 2 had malignant fibrous histiocytoma (MFH), and 1 had a rhabdomyosarcoma.

Table 2. Cytogenetic analysis of postradiation sarcomas[#]

Patient	Histological type	Karyotype
5	MFH	5 abnormal, non analyzable metaphases <3n-4n>/46,XX [1]
6	fibrosarcoma	42~45,Y,inv(X)(q13q28),add(2)(p21),add(3)(q13),der(6;12) (p10;q10),der(7)t(7;9) (p13;q11),der(8)(t(8;?13)(q23;q11~12), -9,der(9)?t(7;9)(p13;q11),-10,add(11)(p11), -12,-3,add(13) (q32),i(15)(q10),der(19)(t(1;19)(q21;q13),-20,-21,add(21) (p11), -22,der(?)t(?;14)(?;q11),+mar1,+mar2,+mar3 [cp9] /41~43,-Y,inv(X)(q13q28), ?t(1;11)(q23;q23),der(1)t(1;15) (p?31;q?21)add(1)(q21),add(2)(p21), t(2;5)(p13;q13),der(3) t(1;?;3)(q25;?;q12),der(6;12)(p10;q10), t(7;9) (p13;q11),-8, -10,der(11)t(11;15)(p12~14;q14~21),-12,-13,der(14)t(8;14) (q11.2~13;q22~24)t(8;13)(q23;q11~12),add(15)(p11), der(15) t(1;15)(p?31;q?21),del(16)(q?21q?24),der(19)t(1;19)(q21;q13), -20,-21,add(21)(p11),-22, +mar1,+mar2,+mar3,+mar4 [cp5]/ 46,XY [11]
11	NOS	62~72,XX,-X,-1,-2 , add(2)(p21)del(2)(q33),del(2)(q35), add(3)(q11) or der(?)t(?;3)(?;p11),del(3)(p14),+del(3)(p14),-4, -5,add(6)(p12)x2,add(6)(q13), der(6)add(6)(p12)add(6)(q2?1), +7,-8,-9,?dup(10)(q23q24 or q24q25), +?dup(10)(q23q24 or q24q25),del(11)(q21q23),-12,add(12)(p13), der(12)t(12;14) (p11;q11),-13,-13,?add(13)(q31), -14,-14,-15,-16,-16,add(16) (q21),-17,-18,-19,add(19)(q13),-20,+21,+22,+der(?)t(?;6) (?;p11)x2,+r,+mar1x2,+mar2x2,+mar3x2,+4~11mar,~2dmin [cp7]
12[*]	NOS	46,X,add(X)(p11),?der(X;16)(q11;p13),-3,add(4)(p?), der(11)t(11;12)(p15;q13), -12,+ mar1,+mar2 [cp2]/46,XX [1]

[#] Cytogenetic nomenclature according to the International System for Human Cytogenetic Nomenclature (1995); F. Mitelman (Editor), S. Karger Publishing, Basel, Switzerland

[*] Described before by Molenaar et al in Lab. Invest., *60*: 266-274, 1989 under a previous nomenclature system (ISCN 1985)

All 3 of the angiosarcomas developed after breast conservative treatment for breast cancer. The rhabdomyosarcoma had developed 21.5 years after treatment for hereditary retinoblastoma. Despite additional immunohistochemical staining, the histological type could not be specified in 6 cases, referred to as sarcoma NOS. In 4 cases a karyotype of the postradiation sarcoma was established (Table 2). This revealed complex karyotypes known to be characteristic for such tumors.[21] In 1 case, 5

abnormal metaphases with clear chromosomal abnormalities were seen, but chromosomes were not individually analyzable.

Follow-up. The median survival after diagnosis of the postradiation sarcoma was 17.5 months for 15 evaluable patients (range, 2–161 months). Twelve patients were either alive with disease or dead of disease, with a median overall survival of 13 months (range, 2–58 months) after being diagnosed with a postradiation sarcoma. Eleven patients with recurrent disease had local failure, whereas 4 also had distant metastases. One patient, diagnosed with a postradiation angiosarcoma, suffered from distant disease without a recurrence at the primary site. Three patients were disease-free at 43, 60, and 161 months after treatment for postradiation sarcoma. One patient was not evaluable for clinical follow-up because no clinical record was available anymore.

KIT expression in postradiation sarcomas. The results on KIT scoring are given in Table 1. Fourteen of 16 tumor samples were positive for KIT (88% of cases). Eight specimens demonstrated ≥80% immunoreactive tumor cells (50% of cases). Three samples had 50–80% positive tumor cells (19% of cases). In 3 samples <50% of positive tumors cells were observed (19% of cases). Two samples revealed no KIT-positive tumor cells (13% of cases), yet immunoreactive mast cells were present.

KIT expression per histological type of postradiation sarcoma. Two of 3 angiosarcomas revealed >80% positive tumor cells, whereas the third had few solitary positive tumor cells. Both MFH were strongly positive for KIT (>80% of the tumor cells). The 2 fibrosarcomas showed 50–80% positive tumor cells. The 1 postradiation rhabdomyosarcoma had >80% positive tumor cells. Both osteosarcomas revealed positive tumor cells, but in each specimen this totaled <50%. Of the 6 sarcomas NOS, 3 were strongly positive (>80%), 1 had 50– 80% positive tumor cells, whereas 2 were negative.

KIT expression in spontaneous soft tissue sarcomas. Twenty-three spontaneous soft tissue sarcomas of comparable histological type were studied for KIT expression as well (Table 3). In contrast to the postradiation sarcomas, only 5 (22%) of these tumors were KIT-positive. None had ≥80% KIT-positive tumor cells. Three samples revealed 50–80% KIT-positive tumor cells: 2 were angiosarcomas and 1 was a sarcoma NOS. Two MFH showed focal immunoreactive tumor cells totaling <50% of all of the tumor cells in these samples.

Table 3. KIT expression in spontaneous soft tissue sarcomas

Histological type	Negative	<50% positive tumor cells	50-80% positive tumor cells	>80% positive tumor cells	Total
MFH	4	2	0	0	6
Rhabdomyo-sarcoma	6	0	0	0	6
Angiosarcoma	1	0	2	0	3
Fibrosarcoma	2	0	0	0	2
Sarcoma NOS	5	0	1	0	6

Number of samples categorized for histological type and KIT expression

Exon 11 of the *c-kit* gene. Direct sequencing of exon 11 of the *c-kit* gene could be performed in 13 cases; all of these tumor specimens were obtained in 1994 or later. In 3 cases the PCR to obtain an adequate DNA sample had failed; these specimens were paraffin-embedded and dated 1993 or before. None of the analyzed samples revealed a mutation in exon 11.

Discussion

Previous radiotherapy is a recognized risk factor for the development of sarcomas.[22] Amendola *et al.*[23] estimated an incidence of 0.09–0.11% after all cases of radiation therapy. Recent reports suggest an increasing incidence, possibly because of the introduction of techniques such as breast conservative treatment for breast carcinoma.[24] Three patients of the current series underwent breast conservative treatment; all 3 were diagnosed with an angiosarcoma. One patient, who had been treated at the 2 years of age with bulbar enucleation and subsequent irradiation for hereditary retinoblastoma, developed a rhabdomyosarcoma after almost 22 years. Hereditary retinoblastoma is a recognized additional risk factor for postradiation malignancies, both carcinomas and sarcomas. Nevertheless, the presentation of a postradiation rhabdomyosarcoma as observed in this case is rare.[25]

Prognosis for patients diagnosed with a postradiation sarcoma is generally poor.[26] Lagrange *et al.*[14] reported a median survival of 23 months in a series of 80 patients. Patients from the current study survived a median period of only 17.5 months. Radical resection results in a relatively favorable outcome with up to 39% 5-year survivors, but may not be

feasible.[14] Distal location of the postradiation sarcoma is a favorable factor, as it increases the possibility for radical surgery.[27] In the current study, only 3 of 15 evaluable patients were disease-free 43, 60, and 161 months after diagnosis. The latter 2 patients had distally located sarcomas (primary site: groin and upper leg), and both underwent radical surgery. Twelve patients had recurrent disease, which was accompanied by a median survival of only 13 months. Local recurrence was observed in 73% of the patients and appears to be a major cause of death. Distant disease was observed in 33% of the cases and was in all but 1 case accompanied by a local recurrence.

Mertens *et al.*[21] described the complex karyotypes found in 10 newly described postradiation sarcomas and 8 cases published previously. The complexity of the karyotypes was in concordance with our findings; the reported high frequency of rearrangements of chromosome 3 was also observed in 3 of 4 cases of the present series. However, no distinctive cytogenetic aberrations are known to be specific for (a subset of) postradiation sarcomas.

Five patients of the current study with postradiation soft tissue sarcoma were treated with anthracycline-based chemotherapy: 4 of them died of disease within 14 months after treatment. Only 1 patient who received adjuvant chemotherapy after radical surgery is alive after 161 months without evidence of disease. To date, no randomized studies have been performed to reveal the value of chemotherapy for postradiation sarcomas, which can be explained by the extreme rarity of the disease. Anecdotal reports mostly concern patients with disease in an advanced stage, a situation in which conventional chemotherapy appears to be ineffective.[1,28,29] Favorable results have been reported for postradiation osteosarcomas after methotrexate-based treatment, comparable with the spontaneous osteosarcomas.[30-32] In the current series, 2 patients had a postradiation osteosarcoma. One was treated with methotrexate plus cisplatin followed by surgical resection and is alive 43 months after diagnosis without overt disease.

KIT tyrosine kinase activity has been linked to the genesis of GIST. Rubin *et al.*[7] reported that GIST (benign, borderline, and malignant) all demonstrated elevated levels of KIT tyrosine kinase activity, whereas 92% harbored a mutant *c-kit* gene. Inhibition of KIT by the small-molecular agent imatinib mesylate renders considerable response rates in patients with metastasized malignant GIST.[11] To date, it is unknown whether other cancer types are driven by KIT-mediated cell signaling and might therefore benefit from inhibition of KIT activity. In the current series of

postradiation sarcomas, 14 of 16 cases were positive for KIT expression. Ten of these 14 positive samples revealed >50% immunoreactive tumor cells. Eight samples had even >80% positive tumor cells. KIT expression was not only evident in postradiation angiosarcomas, but also in other histological types. KIT expression was considerably more pronounced in postradiation sarcomas compared with a group of non-postradiation, non-GIST sarcomas. Hornick and Fletcher [33] also found limited expression of KIT in spontaneous soft tissue sarcomas when using the same antibody. In the current group of 23 spontaneous sarcomas, 2 angiosarcomas and 1 sarcoma NOS revealed >50%, but not >80% positive tumor cells. Angiosarcomas, also when spontaneously arising, were reported to express KIT in a substantial amount of cases.[2] Two spontaneous MFH revealed limited KIT expression, and 2 were negative, in contrast with the 2 postradiation MFH with strong and diffuse (>80%) positive tumor cells. Spontaneous rhabdomyosarcomas and fibrosarcomas were found to be KIT-negative, similar to the findings of Hornick and Fletcher.[33] Four of the 5 spontaneous sarcomas NOS, high-grade tumors with insufficient characteristics for specific histological typing, were KIT-negative.

Whether postradiation sarcomas will respond to KIT inhibition remains to be established. In malignant GISTs, the responsiveness to imatinib mesylate depends on the presence of specific mutations in the *c-kit* gene. Heinrich *et al.*[12] found a far better response in malignant GISTs bearing an activating mutation in exon 11 of the *c-kit* gene. Their results prompted a molecular analysis on this exon for the postradiation sarcomas. However, 0 of 13 analyzed samples revealed a mutation in exon 11. Our results on exon 11 status suggest that different roles of KIT function exist between postradiation sarcomas and malignant GIST. An anticancer effect of KIT inhibition may be expected when it actually mediates an oncogenetic drive. This may still involve mutational activation of KIT, yet in that case it is more likely to occur in regions other than exon 11. Deregulated autocrine or paracrine loops between KIT and its ligand provide an alternative mechanism in which imatinib mesylate or other KIT inhibitors may interfere.[34]

The presented results warrant additional study on KIT inhibition in patients diagnosed with primarily irresectable postradiation sarcomas. To date, it is unclear whether KIT inhibition will demonstrate activity against postradiation sarcomas. However, because of the rarity of such tumors, this report aims to raise the awareness to a potential treatment against these typically aggressive and resistant tumors.

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Chapter 10

Summary, conclusions and perspectives

Samenvatting, conclusies and vooruitzichten

Summary, conclusions and perspectives

Soft tissue sarcomas are rare malignancies originating from mesenchymal origin. They may occur at any age, but the incidence increases with age: about 50% of the patients are over 60 years of age. A distinct peak incidence is made up by embryonal rhabdomyosarcomas that mostly afflict children at age less than 5 years. The local treatment of soft tissue sarcomas is currently well defined: surgical resection, followed by adjuvant radiotherapy when indicated by the microscopic resection margin, high tumor grade and tumor size. Still, metastasis is common in soft tissue sarcomas: 10% of patients initially present with metastases and up to 30-40% of all patients with high-grade, localized tumors progress to metastatic disease. Metastatic disease is seldom amenable to curative treatment. The current role for chemotherapy in metastatic soft tissue sarcoma is palliative, except for pediatric rhabdomyosarcoma and extraskeletal Ewing's sarcoma / primitive neuroectodermal tumor, for which cure can be achieved. New, effective treatment strategies are required, especially to deal with metastatic soft tissue sarcomas in adults. Soft tissue sarcomas cover a broad range of histological types, some of which are additionally divided into subtypes. The distinction between these types has traditionally been based on microscopic evaluation of tumor morphology. While this still is the cornerstone of the histological classification, immunohistochemistry has widely contributed to assign the specific type of soft tissue sarcoma. Detection of genetic anomalies is helpful and sometimes determinative in designating histological type, e.g. t(12;16)(q13;p11) for myxoid liposarcoma, t(2;13)(q35;q14) for alveolar rhabdomyosarcoma, t(X;18)(p11;q11) for synovial sarcoma and t(11;22)(q24;q12) for (extraskeletal) Ewing's sarcoma. The need to differentiate between the respective types of soft tissue sarcomas is enforced by the considerable differences that exist between the histological types, including their responsiveness to chemotherapy. However, in current clinical practice, the histological type of soft tissue sarcoma seldom influences the choice of chemotherapy.

In C**hapter 2** the factors are reviewed that are associated with the occurrence of metastases from soft tissue sarcomas. This is the basis to predict patient survival and for treatment planning.

In this chapter, existing options for the treatment of metastatic disease are also discussed. While surgery can cure strictly selected patients, the majority of patients with metastases can only be treated with

chemotherapy. Unfortunately, chemotherapy seldom cures patients in this stage and improves survival only in a limited number of patients. Failure to chemotherapy may in part be caused by so-called multidrug resistance (MDR). MDR can be conveyed by specific proteins like P-gp, MRP1 and LRP and it appears that most types of soft tissue sarcoma show profound expression of these proteins. Based on results from laboratory experiments, MDR modulating agents were assumed to form a breakthrough in circumventing failure to chemotherapy. However, results from clinical studies were disappointing as the modulating agents often caused unacceptable adverse effects. Nonetheless, promising results of a recent study with a second generation P-gp/MRP1 inhibitor in anthracycline-refractory soft tissue sarcomas gives new impulse to further investigation on this strategy. New, third-generation, inhibitors of P-gp function are underway and may bring success in reversing drug-resistance in soft tissue sarcomas.

Apoptosis-inducing cytokines and tyrosine kinase inhibitors have demonstrated anticancer activity, in both laboratory and clinical studies. These non-traditional agents are not directed against DNA, but bind specific membrane receptors, thereby generating pro-apoptotic signals. The role of these agents in soft tissue sarcoma treatment is extensively discussed in Chapter 2.

Chapter 3 presents the results of an immunohistochemical assessment of P-gp, MRP1 and LRP expression in 141 primary soft tissue sarcomas, unexposed to chemotherapy. These soft tissue sarcomas were evaluated according to their histological type and grade. P-gp, MRP1 and LRP were expressed in the majority of soft tissue sarcomas, but their expression was disproportionate over the different histological types. Sometimes different expression was observed between subtypes, most obvious for the absent LRP expression in the myxoid liposarcomas, contrary to the other subtypes of liposarcoma. For the overall group, MRP1 and LRP (but not P-gp expression) were found to be correlated with tumor grade. While differences in clinical behavior can be observed within the heterogeneous group of soft tissue sarcomas, it was suggested that MDR-protein expression might have a role in this. Thereby, this study provides additional arguments to the concept of studying the histological types and grades of soft tissue sarcomas individually, instead of lumping them together.

Chapters 4 and 5 share the rhabdomyosarcoma histological type as study subject. Children with rhabdomyosarcomas are known to have a more advantageous prognosis after multimodality treatment, including

chemotherapy, than adult patients. It was therefore suggested that MDR proteins might play a role and that these were expressed differently in rhabdomyosarcomas obtained from pediatric and adult patients.

Chapter 4 describes that LRP (but not P-gp or MRP1) was relatively over-expressed in the adult rhabdomyosarcomas. Moreover, the expression of LRP was positively correlated with increasing age at diagnosis. In line with these finding, it was suggested that the more profound LRP expression in tumors of adult patients might decrease their sensitivity to cytotoxic drugs. The rare alveolar rhabdomyosarcoma subtype took a distinct position with its low to absent LRP expression. Other mechanisms than LRP expression appear to be responsible for the resistant phenotype for this particular subtype. However, due to the limited number of alveolar rhabdomyosarcomas, no solid conclusions could be drawn.

In **Chapter 5,** alterations in the expression of MDR proteins P-gp, MRP1 and LRP before and after chemotherapy are described in a series of 13 rhabdomyosarcomas. The residual lesion after chemotherapy appeared significantly better differentiated than the tumor in its initial chemotherapy-naive state. It was found that this differentiation was accompanied by an increase in LRP expression. Contrary, P-gp or MRP1 expression did not change significantly after chemotherapy. Interestingly, in both the untreated and the treated samples, LRP was expressed primarily in the more differentiated tumor cells. On the basis of these findings, it was hypothesized that chemotherapy might have spared the more differentiated cells due to their expression of LRP. Also, the expression of LRP appeared to be induced by chemotherapeutic treatment.

In **Chapter 6**, the expression of P-gp, MRP1 and LRP in related samples of primary tumors and metastases is outlined. Metastatic soft tissue sarcoma in adults has a 20% to 30% response rate to cytotoxic treatment. To date it is unknown whether soft tissue sarcoma metastases are more resistant than their primary counterparts. Several mechanisms can be held responsible for the resistance to cytotoxic agents, including the functioning of the studied MDR associated proteins. Unexpectedly, the expression of P-gp was found to be decreased in metastases compared to their corresponding primary tumors. While ahead of being able to draw definitive conclusions, this finding suggests that the presence of metastases does by itself not imply a more profound resistance phenotype.

Chapter 7 analyzes the expression of P-gp, MRP1 and LRP in 37 locally advanced extremity soft tissue sarcomas, not manageable with radical surgery at time of presentation. All tumors were treated with a hyperthermic isolated limb perfusion (HILP), with administration of tumor

necrosis factor-α (TNF-α) and melphalan at high concentrations. This procedure aims to enable radical surgery at a later stage. Tumor samples were taken before and after HILP in order to assess potential changes in MDR protein expression. On the whole, it was found that expression was not altered after HILP. What might have interfered with the results are the number of tumors with complete response and the time between tumor sampling. First, in those cases with a complete histological response, no viable tumor tissue was left, inherently disclosing no information on MDR expression after treatment. However, especially these tumors with a complete response to chemotherapy would have been interesting from a mechanistical point-of-view. Second, acute alterations in expression might have been missed because of the prolonged time between obtaining the pre-treatment sample and the post-treatment sample.

A recent study by Stein et al with sequential sampling of 14 soft tissue sarcomas during and shortly after HILP came to similar findings and conclusions on P-gp and MRP1 expression. LRP appeared to have increased shortly after exposure to TNF-α and melphalan, as observed by mRNA and protein expression.

Chapter 8 presents an in vitro study on the effects of the 'standard' cytotoxic drugs doxorubicin and activated ifosfamide with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in KYM-1, a rhabdomyosarcoma cell line sensitive to TNF-α , its five-fold TNF-α sensitive subline KD4 and its over 150-fold TNF-α resistant subline 37B8R. Like TNF-α, TRAIL also induces apoptosis. Resistance to TRAIL has been demonstrated, but can be circumvented by combinations of TRAIL with conventional cytotoxic agents, *in vitro*. In the tested cell lines, sensitivity and resistance to TNF-α and TRAIL paralleled: TRAIL alone was effective in inducing cell death in KYM-1 and even more in KD4, while 37B8R was largely refractory to TRAIL-mediated apoptosis. The enhanced cytotoxic effects of combining doxorubicin with TRAIL became particularly apparent in the 37B8R cell line. TRAIL resistance was independent from membrane expression of TRAIL receptors DR4/DR5 and DcR1/DcR2. The combination of TRAIL plus doxorubicin showed synergistic effects on cytotoxicity and apoptosis in all three cell lines. The combination of TRAIL plus 4-hydroxy-ifosfamide showed to be additive in most of the times. At this moment, clinical trials with TRAIL are commencing. The results of this study suggest that TRAIL can be effective in killing soft tissue sarcoma cells. Moreover, the addition of cytotoxic drugs, in particular doxorubicin to TRAIL can be of value in circumventing resistance to TRAIL.

Chapter 9 concerns sarcomas arising in previously irradiated tissues, the so-called postradiation sarcomas. The presented study dealing with 16 cases of postradiation sarcomas illustrates the difficulty of managing these aggressive tumors. Additional radiotherapy is often impossible, because of the risk for severe tissue damage. Chemotherapy is generally ineffective against these tumors. Therefore, radical surgery is crucial, but can in fact be difficult to achieve. With a median overall survival of less than 18 months, prognosis was extremely poor.

Contrary to spontaneous soft tissue sarcomas, the vast majority of the 16 postradiation sarcomas expressed the c-KIT receptor tyrosine kinase. c-KIT is the target for imatinib mesylate, an agent already proven to be effective against c-KIT-expressing sarcomas of the digestive tract, the gastrointestinal stromal tumors (GIST). None of the evaluable samples of the postradiation sarcomas revealed mutations of exon 11 of the *c-kit* gene, while the presence of an exon 11 mutation is correlated to the effect of imatinib mesylate in malignant GIST. Future study should determine the value of imatinib mesylate or alternative inhibitors of c-KIT in the treatment of postradiation sarcomas.

General considerations

Recent literature and contents of this thesis emphasize the importance of histological type and grade on the management of soft tissue sarcomas. The progress of analytical techniques is helpful in making relevant distinctions between soft tissue sarcomas.

Notwithstanding significant advances in local treatment, many soft tissue sarcoma patients suffer the consequences of uncontrollable disease. The continuing challenge is to develop effective treatment strategies from the increasing knowledge brought forth by basic research, of which especially patients with metastatic lesions should benefit. The search for oncogenic pathways appears a promising approach in finding new systemic treatments and is aided by micro-array technology. This technique allows the simultaneous assessment of thousands of genes from a single tumor sample at an unprecedented rate, enabling researchers to uncover genes that govern sarcomagenesis and metastasis [1]; these are potential targets for new treatments.

Future perspectives

To improve the management over soft tissue sarcomas, the key elements that govern their biological behavior are the subject of intensive research. In order to detect tumorbiological elements, immunohistochemistry was applied in most of the studies presented in this thesis. When interpreting any immunohistochemical staining, one has to realize that the outcome is a function of various variables. Expression of P-gp in soft tissue sarcomas was assessed in several studies of the current thesis and it appears striking that various investigators have reported levels of 0% to virtually 100% tumor cells in soft tissue sarcoma samples being P-gp positive (references on these studies are listed in Chapter 3). Next to methodological causes for this obvious discrepancy, there exists another issue that is typical for soft tissue sarcomas, namely the inclusion of different histological subtypes. Therefore, not only a consensus recommendation on the applied technique and scoring is required [2], careful evaluation of the included histological type is also required before comparing the individual studies. In the studies covered by this thesis, immunohistochemistry was performed to a uniform protocol and small inter-observer variability was noted. In Chapter 3, the 141 cases of soft tissue sarcoma were sorted by histological type and grade, while Chapter 4 and 5 deal with rhabdomyosarcoma as the single type.

There is also rising concern for the immunohistochemical detection of c-KIT.[3] Immunohistochemical staining of c-KIT is yet in an early phase. Contradictory results have been obtained within single studies when using different antibodies to c-KIT.[4,5] The Groningen Sarcoma Group uses the rabbit polyclonal anti-c-KIT antibody A4502, manufactured by DAKO. This antibody was commonly used for accrual of patients with suspected malignant GIST in the phase I imatinib mesylate studies.[6] The Groningen Sarcoma Group favors an epitope retrieval before exposing the tissue samples to the antibody, thereby obtaining apparently specific results, as has been detailed in Chapter 9. Still, others advocate a procedure without epitope retrieval. When comparing immunohistochemical studies on c-KIT, differences in the methodology can often be observed. These differences, e.g. the choice of a buffering solution, might greatly influence the outcome.

For the anti-c-KIT antibody A4502, this is demonstrated on the website of the collaborating Scandinavian laboratories of Pathology: http://www.nordiqc.org/Assessments/Run7/CD117-as7.htm, (last update: October 2003).

In the oncologic setting, immunohistochemistry can be applied for

tumor classification, prognostigational purposes and to predict treatment response. P-gp, MRP1, LRP and c-KIT are involved in tumor biology, and all (but LRP) serve as potential targets to attack soft tissue sarcomas. This emphasizes the importance of a reliable methodology to detect their expression. With regard to c-KIT, its presence alone is insufficient to predict the response toward c-KIT-targeted treatment; information about the mutational status of the *c-kit* gene provides additional information to this matter. Therefore, one has to bear in mind that molecular alterations of the target can also affect the success of treatment. Additional techniques at a molecular level (e.g. DNA analysis) will certainly become part of near-future clinical work-up.

Systemic therapy in soft tissue sarcoma treatment: "Something old, something new, …"

Doxorubicin, is it worthwhile? Systemic treatment of malignancies is required when local treatment by surgery and radiation therapy cannot be applied. While most studies designate locally advanced and metastatic soft tissue sarcoma as an incurable disease, a recent study identified a subgroup of patients who survived a 5-years period after doxorubicin-based treatment.[7] This remarkable outcome legitimates the use of doxorubicin-containing chemotherapy in advanced soft tissue sarcomas. Still, it would be of tremendous clinical value when the patients with potentially sensitive tumors could be identified in advance. In this regard, it would be challenging to investigate whether the favorably responding tumors possess less pronounced expression of (functional) MDR-associated proteins.

Conventional agents other than doxorubicin or ifosfamide. Although doxorubicin and ifosfamide are the most widely used agents in soft tissue sarcoma treatment, other conventional agents might also be of value. Paclitaxel appears effective in angiosarcomas of head and neck [8], but not for other major histological types of soft tissue sarcoma [9], while docetaxel combined with gemcitabin was reported to be beneficial for patients bearing uterine leiomyosarcomas in a single study.[10]

Preliminary data of soft tissue sarcoma studies suggest that liposomal doxorubicin is equally active to standard doxorubicin, while having fewer side-effects.[11] This particular formulation can therefore be considered for those patients who are at risk for of intolerable cardiotoxicity from

standard doxorubicin.

Trofosfamide is an orally available drug, structurally related to ifosfamide and cyclophosphamide, and has been demonstrated to be relatively well-tolerated and active in soft tissue sarcoma patients.[12-15] The relatively favorable profile of adverse reactions and its oral availability make trofosfamide an interesting drug, especially for elderly patients.

Ecteinascidin-743. Ecteinascidin-743 (ET-743, trabectidin) is a novel anticancer agent derived from the sea squirt *Ecteinascidin turbinata*. ET-743 binds to DNA and inhibits subsequent DNA transcriptional activation. In vitro research has demonstrated the cytotoxic effects of ET-743 on soft tissue sarcoma cell lines derived from diverse histological types.[16] Also in the clinical situation, ET-743 appears an active agent in soft tissue sarcoma treatment.[17-19]

Recently, it has been reported that ET-743 can also inhibit the activation of the P-gp (MDR1) gene.[20] This offers the opportunity to modulate P-gp-mediated drug resistance with ET-743. The proof-of-concept of this mechanism has been demonstrated by *in vitro* experiments, in which soft tissue sarcoma cells were pretreated with ET-743, and started to accumulate doxorubicin and became more sensitive to doxorubicin.[21]

Thiazolidinediones. Thiazolidinediones are a category of synthetic ligands to the peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is a nuclear hormone receptor that is a key regulator of adipose cell differentiation. However, besides this physiological role, PPAR-γ appears also to be involved in the oncogenesis of liposarcomas. When human liposarcoma cells were exposed to the thiazolidinediones pioglitazone, apparently normal adipocytic differentiation occurred.[22] A report on three cases of liposarcoma in vivo showed differentiation of tumor cells to morphologically mature adipocytes during treatment with the thiazolidinedione troglitazone.[23] Because of its potential hepatotoxicity, troglizatone has been withdrawn by its manufacturer; the related rosiglitazone was used in a subsequent clinical study (NCI-G99-1629 of the National Cancer Institute, Bethesda, USA). As of yet, no results of this study have been reported, apart from preliminary data in which no radiological responses were observed. While it is too early to foresee the achievability of combination therapies, the *in vitro* antineoplastic effects of thiazolidinediones with TRAIL [24,25] or non-steroidal anti-inflammatory drugs [26] might point out a new lead.

Modulators of drug efflux pumps. Despite promising results from laboratory experiments with inhibitors of drug efflux pumps (mainly P-gp and MRP1), these agents have largely failed in the actual treatment of solid tumors. However, recently, Bramwell et al claimed an antitumor effect of VX-710 (Biricodar™), a modulator of P-gp and MRP1 function, in combination with doxorubicin in formerly doxorubicin-refractory sarcomas.[27] These results require confirmation by additional studies, with careful assessment of toxic side-effects. Still, this second-generation modulating agent may be able to circumvent classical MDR in soft tissue sarcomas. At present, third-generation modulators have reached clinical trials and might be of value.[28]

While P-gp is involved in drug resistance towards conventional cytotoxic agents, new evidence emerges that P-gp might also diminish the effects of new agents like imatinib mesylate.[29,30] Therefore, it would be of interest whether GIST would become more sensitive or regain sensitivity to imatinib mesylate, when combined with a P-gp modulator. As malignant GIST are characterized by abundant expression of P-gp [31], this appears a interesting approach.

Inhibitors of receptor tyrosine kinase activity. Signal transduction is the process of converting extracellular signals to an intracellular response. An important fashion of signal transduction is binding of ligands to receptor tyrosine kinases, controlling essential cellular processes, including proliferation, differentiation and apoptosis. Deregulation of receptor tyrosine kinase activity results in aberrant signal transduction, which may lead to the malignant transformation of cells. The enzymatic activity of receptor tyrosine kinases, located at the intracellular domain, is the transfer of a phosphate-group from adenosine triphosphate (ATP) to the specific substrate. This step can be inhibited by small-molecular agents that comprise a whole new class of anticancer agents.

For soft tissue sarcomas, several receptor kinases come into view, as they are involved in sarcomagenesis. For some of these, c-KIT, platelet-derived growth factor (PDGF-R) and vascular endothelial growth factor-receptor (VEGF-R), small-molecular inhibitors are already available.

In malignant GIST, the c-KIT receptor tyrosine kinase can become autonomously activated due to mutations of the *c-kit* gene. Imatinib mesylate, initially introduced to block Bcr-Abl tyrosine kinase in chronic myeloid leukemia, inhibits c-KIT and PDGF-R activity as well. The first report on the anticancer effect of imatinib mesylate in a patient with metastatic malignant GIST was astounding.[32] Later, Heinrich et al

demonstrated that in malignant GIST, the tumor response depends on the mutational status of the *c-kit* gene, with exon 11 as the most favorable factor.[33] Those with malignant GIST that bear no or other mutations of the *c-kit* gene are less responsive to imatinib mesylate.[34] About 15% of GIST that bear an exon 11 mutation are intrinsically resistant to imatinib mesylate. In addition, initially sensitive GIST can acquire resistance to imatinib.[35] GIST that are resistant to imatinib mesylate may however still be sensitive to c-KIT inhibition by alternative agents, like SU11248.[36] Next to inhibition of c-KIT, SU11248 also inhibits PDGF-R, VEGF-R and FLT3 (fms-related tyrosine kinase 3). Therefore, the effect of this agent may also be mediated through the blockade of signaling mediated by (one of) these receptor tyrosine kinases.

The effect of imatinib mesylate in GISTs warrants the identification of other tumor types appropriate for this agent. Most types of soft tissue sarcoma that bear no immunohistochemically detectable expression of c-KIT appear no suitable candidate for imatinib mesylate treatment.[37] Still, preliminary data suggest that patients with dermatofibrosarcoma protuberans, a soft tissue sarcoma type that is located in the superficial layers, may also benefit from imatinib.[38] These tumors are characterized by expression of a COL1A1-PDGF β fusion protein, leading to autocrine stimulation of PDGF-R β. Blockage of PDGF-R β activity can lead to apoptosis of dermatofibrosarcoma protuberans cells.[39] Data from a xenograft model suggest that imatinib mesylate might also be of value for (extra-skeletal) Ewing's sarcoma.[40] In this case, the effect appears to be mediated through the abrogation of unbalanced c-KIT activity, generated by autocrine binding of its natural ligand.

The clinical success of receptor tyrosine kinase inhibitors will certainly not be restricted to imatinib mesylate targeting of c-KIT in malignant GISTs. Other receptor tyrosine kinase inhibitors are under construction or are already available, aimed at c-KIT as well as other tyrosine kinases. For example, SU6668 can inhibit VEGF-R, PDGF-R and FGF-R (fibroblast growth factor receptor). VEGF-R has been demonstrated in angiosarcomas and it appears challenging to disclose the effect of inhibiting its activity in this tumor type.[41]

At this time, EORTC phase II trial 62022 is accruing patients with synovial sarcomas that express epithelial growth factor receptor 1 (EGF-R1), with the intention to treat them with gefitinib (ZD 1839, Iressa™, Astra Zeneca), a small molecule that inhibits epidermal growth factor-receptor 1 (EGF-R1).

It is difficult to predict what kind of long-term complications are to be

expected from these new agents. At this moment, the first patients treated with imatinib mesylate are about two and a half years in treatment protocols. Serious side effects involve tumor hemorrhage in malignant GIST and depression of hematopoiesis mainly in chronic myeloid leukemia. Other remarkable side effects the like the regain of pigmentation in formerly gray hairs, probably involving c-KIT expression in melanocytes, are also becoming evident.[42]

TRAIL. Tumor necrosis factor-related apoptosis inducing ligand, or TRAIL, holds the promise of effectively inducing apoptosis in tumor cells, while at the same time being harmless to normal cells. The apoptotic activity of TRAIL has been established in tumor cells, including those from mesenchymal origin, but resistance is encountered as well. Therefore, when TRAIL does reach clinical applicability, TRAIL-refractory tumors might be expected. In this thesis, it has been described that combining TRAIL with conventional cytotoxic agents is a way to overcome TRAIL-resistance in soft tissue sarcoma cells. However, it is challenging to find non-toxic agents that have similar effects. In vitro experiments demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs) and thiazolidinediones have synergistic apoptotic effects when combined with TRAIL.[25,43,44]

LRP

Of the proteins involved in MDR, many reports are available on the members of the ATP-binding cassette proteins, which include P-gp and the MRP family. Lung resistance-related protein, or LRP, is an outsider amongst the mediators of MDR: not only in its structure or function, but also for its causality in MDR. For example, transfection of LRP cDNA into tumor cells does not necessarily lead to a drug resistant phenotype.[45] Kitazono et al were the first to provide evidence for a causal role of LRP in MDR.[46] However, other groups have not been capable of similar results.[47] Recently, a study revealed that mice that were genetically altered to lack the LRP gene were not increasingly susceptible to doxorubicin.[48] These results suggest that LRP is not directly involved in MDR. Still, data from clinico-pathological studies, including Chapters 4 and 5 of the current thesis, do link LRP with chemotherapy resistant tumors.[49]

New agents, new endpoints

Cytotoxic agents kill tumors cells, thereby causing a decrease in tumor size. This change in tumor size can be measured through conventional imaging techniques (standard X-ray imaging, spiral computed tomography, magnetic resonance imaging) and is the basis of evaluating the anticancer effect. For sarcomas, the response evaluation criteria in solid tumors (RECIST) can be applied.[50] In the concept of phase II clinical studies, the results of the RECIST criteria are determinative for considering an agent as valuable for further testing. However, new non-cytotoxic agents like the thiazolidinediones and imatinib mesylate do not necessarily have to cause tumor shrinkage in case of a clinical response. The thiazolidinedione troglitazone has been demonstrated to induce maturation of liposarcoma cells [23]: perhaps paradoxically, these liposarcomas gained volume as tumor cells began to store fat droplets as part of their maturation process. Imatinib mesylate decreases the metabolism of GIST cells, and in some instances a decrease in tumor size followed only after a prolonged time.[51] These two examples illustrate the need for alternative end-points when non-cytotoxic agents are used. As these agents halt progress of the disease, progression-arrest rate can be used as a surrogate marker of drug activity. For agents that clearly affect tumor metabolic activity, positron emission tomography using a metabolism-linked tracer like ^{18}F-fluorodeoxyglucose.[52] Sequential tumor biopsy during anticancer treatment offers a direct modality of treatment evaluation, but the invasive aspect with risk of tumor bleeding and tumor spill makes this less attractive. Serum markers indicative for soft tissue sarcomas activity have not yet found clinical use, as their specificity appeared largely unsatisfactory.

Soft tissue sarcomas are intriguing malignancies in their biological characteristics. These malignancies are best managed by an “oncology team approach”, involving clinicians and basic researchers. The current local treatment is well-established and local failure rates are low.[53] Still, 30-40% of the patients develop metastases. The challenge is to develop effective treatments at the cost of tolerable side-effects. Furthermore, the identification of patients that would benefit from adjuvant chemotherapy after local treatment would be a major progress. Only research dedicated to patients with soft tissue sarcomas can bring improvement to current prognosis. The breakthrough of imatinib mesylate in the treatment of malignant GISTs brings motivation to researchers and doctors active in the field of soft tissue sarcomas.

Samenvatting, conclusies en vooruitblik

Weke-delen sarcomen zijn zeldzame maligniteiten van mesenchymale oorsprong. Ze komen voor op alle leeftijden, maar de incidentie neemt toe met het stijgen der jaren: ongeveer de helft van de patiënten is 60 jaar of ouder. Een aparte piek in de incidentie wordt gevormd door embryonale rhabdomyosarcomen, die vooral voorkomen bij kinderen onder de vijf jaar. De lokale behandeling van weke-delen sarcomen bestaat uit chirurgische resectie, gevolgd door radiotherapie in geval van een krappe chirurgische marge, hoge maligniteitsgraad, of een grote tumor. Metastasering komt veel voor bij weke-delen sarcomen: 10% van de patiënten presenteert zich met metastasen, terwijl circa 40% van patiënten met een hooggradig weke-delen sarcoom uiteindelijk metastasen ontwikkelt. Metastatische ziekte is zelden toegankelijk voor in-opzet curatieve behandeling. Chemotherapie heeft bij het gemetastaseerd weke-delen sarcoom een palliatieve rol; alleen in geval van rhabdomyosarcomen van de kinderleeftijd en extraskeletale Ewing sarcomen/ primitieve neuro-ectodermale tumoren kan nog curatie worden bereikt. Effectievere behandelingen zijn nodig, zeker in geval van gemetastaseerde weke delen sarcomen bij volwassenen.

De term “weke-delen sarcoom” omvat in feite een breed scala aan verschillende histologische types, waarvan sommige zijn onderverdeeld in subtypes. Het verschil tussen deze types wordt van oudsher gemaakt op grond van microscopische beoordeling van de tumor morfologie. Hoewel morfologie nog steeds de basis vormt voor de classificatie, heeft met name immunohistochemie een grote bijdrage geleverd aan de nadere specificatie van de afzonderlijke types. Het aantonen van chromosomale afwijkingen is bijdragend, soms zelfs bepalend in de classificatie, zoals t(12;16)(q13;p11) voor het myxoïde liposarcoom, t(2;13)(q35;q14)voor het alveolaire rhabdomyosarcoom, t(X;18)(p11;q11) voor het synoviosarcoom en (t11;22)(q24;q12) voor het (extraskeletale) Ewing sarcoom. Het belang om de types weke-delen sarcomen te onderscheiden wordt duidelijk door aanzienlijke verschillen in het biologisch gedrag en in de gevoeligheid voor chemotherapie. Toch is tot op heden het type weke-delen sarcoom zelden doorslaggevend voor de keuze van de chemotherapeutische behandeling.

In **Hoofdstuk 2** volgt een beschouwing van factoren die geassocieerd zijn met het ontstaan van metastasen van weke-delen sarcomen. Op grond hiervan kan een voorspelling worden gedaan over de levensverwachting en keuze van therapie mede worden bepaald.

In dit hoofdstuk worden ook de bestaande behandelopties voor uitgezaaide ziekte besproken. Chirurgie kan genezing brengen voor een beperkte groep patiënten met metastasen, terwijl het merendeel alleen met chemotherapie kan worden behandelend. Helaas blijkt chemotherapie zeer zelden curatief in dit stadium en slechts een kleine groep patiënten ondervindt een verbeterde overleving. Het falen van chemotherapie kan deels berusten op zogenoemde multidrug resistentie (MDR). MDR kan veroorzaakt worden door eiwitten als P-gp, MRP1 en LRP, waarvan aangetoond is dat zij door de meeste weke-delen sarcomen tot expressie worden gebracht. Afgaande op resultaten van in vitro onderzoek, werd aanvankelijk gedacht dat MDR-modulerende stoffen voor een doorbraak zouden zorgen in het opheffen van ongevoeligheid voor chemotherapie. Echter, de resultaten van klinische studies bleven ver achter bij de hooggespannen verwachtingen, niet zelden door de bijwerkingen van deze MDR-modulerende stoffen. Toch zijn er gunstige resultaten bereikt met een tweede-generatie P-gp/MRP1 remmer in anthracycline-ongevoelige weke-delen sarcomen, zodat er reden is voor verder onderzoek. Nieuwe, derde-generatie, remmers van P-gp functie zijn op komst en kunnen mogelijk zorgen voor het verminderen van MDR in weke-delen sarcomen.

Apoptose-inducerende cytokines en tyrosine kinase remmers blijken effectief als antitumor middel, in zowel laboratorium als klinische onderzoeken. Deze onconventionele middelen zijn niet tegen DNA gericht, maar grijpen specifiek aan op membraan-receptoren van tumorcellen, waarmee een pro-apoptotische signaal gegeneerd wordt. De rol van dergelijke stoffen in het kader van weke delen sarcomen behandeling wordt ook besproken in Hoofdstuk 2.

Hoofdstuk 3 behelst een immunohistochemisch onderzoek naar P-gp, MRP1 en LRP expressie in 141 primaire weke-delen sarcomen, die tevoren niet blootgesteld waren aan chemotherapie. In dit onderzoek werd onderscheid gemaakt naar het histologisch type en de maligniteitsgraad. Hoewel P-gp, MRP1 en LRP in het merendeel van de weke-delen sarcomen tot expressie werden gebracht, bleek dit onevenredig verdeeld over de verscheidene types. Soms was er ook een verschil in expressie tussen subtypes, zoals bijvoorbeeld de afwezigheid van LRP in myxoïde liposarcomen, in tegenstelling tot andere subtypes liposarcomen. Expressie van MRP1 en LRP bleek gecorreleerd met de maligniteitsgraad, terwijl dit niet geval was voor P-gp. Gezien de verschillen in biologisch gedrag tussen de typen weke-delen sarcomen, werd er gespeculeerd over de rol hierin van MDR-eiwitten. Dit onderzoek draagt nadere argumenten aan om

weke-delen sarcomen per type en graad te analyseren, en niet zoals voorheen gebruikelijk als één entiteit.

Hoofdstukken 4 en 5 hebben beide het rhabdomyosarcoom tot onderwerp van studie. Kinderen met een rhabdomyosarcoom hebben gunstiger vooruitzichten na behandeling, inclusief chemotherapie, in vergelijking met volwassenen. Vanwege de rol van MDR eiwitten in de gevoeligheid voor chemotherapie, werd verondersteld dat deze verschillend tot expressie komen in rhabdomyosarcomen van kinderen en volwassenen.

Hoofdstuk 4 beschrijft dat LRP inderdaad relatief tot over-expressie kwam in rhabdomyosarcomen afkomstig van volwassen patiënten. Voor P-gp en MRP1 werden geen verschillen gezien tussen tumoren van kinderen en volwassenen. De expressie van LRP was daarnaast ook gecorreleerd aan de leeftijd bij diagnose. In overeenstemming met deze bevindingen werd gesuggereerd dat de expressie van LRP de gevoeligheid voor chemotherapie in met name rhabdomyosarcomen van volwassenen zou verminderen. Het zeldzame alveolaire subtype nam een aparte plaats in door lage tot afwezige expressie van LRP. Dit zou erop kunnen duiden dat andere mechanismen dan LRP functie verantwoordelijk zijn voor resistentie bij dit subtype. Echter, het aantal alveolaire rhabdomyosarcomen in deze studie was erg laag, zodat hierover slechts voorzichtige conclusies konden worden getrokken.

In **Hoofdstuk 5** worden veranderingen in expressie van P-gp, MRP1 en LRP in 13 rhabdomyosarcomen beschreven, ontstaan na chemotherapie. De resttumor na chemotherapie was beter gedifferentieerd dan de tumor vóór chemotherapie, terwijl differentiatie gepaard ging met een toegenomen expressie van LRP. P-gp en MRP1 waren niet significant veranderd na chemotherapie. Het was opmerkelijk dat LRP voornamelijk tot expressie werd gebracht door de meest gedifferentieerde tumorcellen. Op grond van deze bevindingen werd verondersteld dat de beter gedifferentieerde cellen relatief gespaard waren gebleven voor de effecten van de chemotherapie en LRP expressie onder invloed van de chemotherapie wordt geïnduceerd.

In **Hoofdstuk 6** volgt een uiteenzetting over de expressie van P-gp, MRP1 en LRP in gepaarde coupes van primaire tumoren en hun metastasen. Weke-delen sarcomen bij volwassenen hebben globaal zo'n 20-30% kans op respons op cytotoxische behandeling. Tot op heden is onbekend of metastasen meer resistent zijn dan de primaire tumoren Een aantal mechanismen wordt verantwoordelijk gehouden voor resistentie tegen chemotherapie, waaronder de functie van voornoemde eiwitten.

Tegen de verwachting in werd gevonden dat P-gp in mindere mate tot expressie kwam in de metastasen in vergelijking met de bijbehorende primaire tumoren. Hoewel niet bewijzend, wijst deze studie erop dat de aanwezigheid van metastasen op zichzelf niet inhoudt dat er meer uitsproken resistentie is.

Hoofdstuk 7 beschrijft de expressie van P-gp, MRP1 en LRP in 37 weke-delen sarcomen uitgaande van extremiteiten, allen te zeer uitgebreid voor een adequate chirurgische resectie. Deze tumoren werden voorbehandeld met een hyperthermische geïsoleerde ledemaat perfusie (HILP), waarbij tumor necrosis factor-α (TNF-α) and melfalan in hoge dosering worden toegediend. Dit heeft als doel om de tumor te reduceren, zodat chirurgie in tweede instantie wel mogelijk wordt. Tumor materiaal werd verkregen zowel vóór als ná HILP, en werd onderzocht op de expressie van de MDR eiwitten. Geen van de drie onderzochte eiwitten bleek qua expressie significant veranderd te zijn ná HILP. Een tweetal aspecten die meespelen in de analyse van deze resultaten, is het aantal gevallen met volledige tumor regressie, en de tijdsduur tussen verzamelen van tumor materiaal vóór en ná HILP. In geval van een volledige respons was er geen vitaal tumor weefsel meer tot beschikking, zodat de expressie niet kon worden bepaald, terwijl juist tumoren met volledige a-vitaliteit na behandeling vanuit mechanistisch oogpunt interessant zijn. Ten aanzien van het tweede argument van de tijdsduur tussen afname van tumor materiaal vóór en ná HILP speelt dat snelle en tijdelijke veranderingen gemist kunnen zijn.

Een recent onderzoek van Stein et al met sequentiële sampling van 14 weke-delen sarcomen gedurende en kort na HILP komt tot gelijksoortige bevindingen en conclusies met betrekking tot P-gp en MRP1. Daarentegen bleek dat LRP expressie kort na blootstelling aan TNF-α en melfalan wordt geïnduceerd, zowel op mRNA niveau als op eiwit niveau.

Hoofdstuk 8 betreft een in vitro onderzoek naar de effecten van de standaard cytotoxische middelen doxorubicine en geactiveerd ifosfamide, gecombineerd met tumor necrosis factor -related apoptosis-inducing ligand (TRAIL) in KYM-1, een TNF-α gevoelige rhabdomyosarcoom cellijn, de vijfmaal gevoeligere sublijn KD4 en de 150-voudig ongevoelige sublijn 37B8R. TRAIL is, net zoals TNF-α, in staat om apoptose te induceren. TRAIL-resistentie is eveneens beschreven, maar kan in vitro worden omzeild door combinatie met cytotoxische stoffen. In de onderzochte cellijnen bleek de gevoeligheid voor TNF-α samen te lopen met de gevoeligheid voor TRAIL: TRAIL alleen bleek een sterk cytotoxisch effect te hebben in KYM-1 en nog meer uitgesproken in KD4, terwijl 37B8R

ongevoelig was voor TRAIL. TRAIL resistentie was onafhankelijk van de membraanexpressie van TRAIL receptoren DR4/DR5 en DcR1/DcR2. De combinatie van TRAIL met doxorubicine werkte synergistisch voor alle drie cellijnen, zowel in cytotoxiciteitsproeven als in apoptose-proeven. Het versterkte cytotoxische effect van de combinatie van doxorubicine met TRAIL werd vooral duidelijk in de 37B8R cellijn. De combinatie van TRAIL met 4-hydroxy-ifosfamide was meestal additief. Momenteel staan de eerste klinische studies met TRAIL in de startblokken. De uitkomst van deze studie wijst erop dat TRAIL effectief kan zijn in weke-delen sarcomen cellen, en dat doxorubicine in vitro in staat is om TRAIL-resistentie te overwinnen.

Hoofdstuk 9 heeft tot onderwerp sarcomen die in bestraald gebied optreden, ofwel postradiatie sarcomen. Zestien gevallen van postradiatie sarcomen worden beschreven, waarbij de behandelingsmogelijkheden beperkt blijken. Additionele radiotherapie is vaak onmogelijk omdat de maximale bestralingsdosis voor het aangedane lichaamsgebied al bereikt is. Ook chemotherapie is vaak weinig effectief. Daarom is radicale chirurgie van het grootste belang, maar is niet altijd mogelijk. Met een mediane overleving van minder dan 18 maanden, was de prognose voor de patiënten uit deze studie uitgesproken slecht.

Anders dan in sporadische weke-delen sarcomen, bracht de meerderheid van de postradiatie sarcomen het c-KIT tyrosine kinase tot expressie. c-KIT is het aangrijpingspunt voor imatinib mesylaat, waarvan de werkzaamheid bij c-KIT positieve sarcomen van het maagdarmkanaal, maligne gastrointestinale stroma tumoren, (GIST) reeds bevestigd is. Geen van de geanalyseerde postradiatie sarcomen had mutaties in exon 11 van het *c-kit* gen, terwijl dat een gunstige factor is voor de reactie op imatinib mesylaat bij maligne GISTen. Toekomstig onderzoek zal duidelijk moeten maken of c-KIT ook een aangrijpingspunt voor behandeling is van de postradiatie sarcomen.

Algemene overwegingen

Recente publicaties, alsook het voorliggende proefschrift, benadrukken het belang van het indelen van weke-delen sarcomen op basis van het histologische type en de maligniteitsgraad. Ontwikkelingen in analytische mogelijkheden dragen bij om deze indeling te kunnen maken.
Ondanks adequate locale behandeling krijgen veel patiënten te maken met uitzaaiingen. Eén van de uitdagingen op het gebied van weke-delen

sarcomen wordt dan ook gevormd in het vinden van effectieve behandelingsmethoden voor patiënten met uitgezaaide ziekte. Het zoeken naar oncogene routes lijkt een veelbelovende benadering hiertoe en wordt gesteund door micro-array technologie. Met deze technologie kunnen gelijktijdig duizenden genen van een enkel stukje tumor worden geanalyseerd. Zo kunnen essentiële genen voor het ontstaan en metastaseren van weke-delen sarcomen worden blootgelegd [1]; dit zijn potentiële doelwitten voor nieuwe behandelingen.

Vooruitblik

Om de behandeling van weke-delen sarcomen te verbeteren is veel onderzoek gaande naar factoren die bepalend zijn voor hun biologisch gedrag. Om tumorbiologisch factoren aan te tonen, werd immuno-histochemie gebruikt in het merendeel van de onderzoeken die in dit proefschrift worden beschreven. Bij het interpreteren van de resultaten van immunohistochemie, dient men zich te realiseren dat de uitkomst van verscheidene variabelen afhangt. Expressie van P-gp in weke-delen sarcomen, zoals ook in het huidige proefschrift geanalyseerd, blijkt in voorgaande studies te kunnen variëren van 0 tot zelfs 100% (een overzicht van referenties kan gevonden worden in Hoofdstuk 3). Behalve methodologische oorzaken voor deze aanzienlijke verschillen, speelt ook een aspect kenmerkend voor weke-delen sarcomen mee: in de verscheidene studies kunnen uiteenlopende histologische types onderzocht zijn. Om onderlinge onderzoeken te kunnen vergelijken is daarom niet alleen standaardisatie van immunohistochemie nodig [2], ook moet duidelijk zijn welke histologische types betrokken waren bij het onderzoek. In de onderzoeken van het huidige proefschrift werd de immunohistochemie op eenduidige wijze uitgevoerd en was een geringe inter-observer variatie. In hoofdstuk 3 werden de 141 gevallen verdeeld naar type en tumorgraad, terwijl in hoofdstukken 4 en 5 alleen het rhabdomyosarcoom type werd onderzocht.

Er is ook veel aandacht voor de immunohistochemische kleuring van c-KIT.[3] Immunohistochemie van c-KIT bevindt zich in een vroeg stadium en er zijn tegenstrijdige resultaten beschreven bij het gebruik van verschillende antilichamen.[4,5] De Sarcomen Werkgroep Groningen gebruikt het konijnen polyclonale antilichaam A4502 gericht tegen c-KIT, van fabrikant DAKO. Dit antilichaam is hetzelfde dat voor de fase I studies gebruikt werd om patiënten met een maligne GIST in de imatinib-studies

te includeren.[6] Verder wordt een *epitope retrieval* procedure uitgevoerd voordat tumor coupes met het antilichaam worden geïncubeerd, waarbij specifieke resultaten kunnen worden behaald. Andere onderzoeksgroepen gebruiken het zelfde antilichaam zonder *epitope retrieval*. Bij vergelijken van de Materiaal en Methode secties tussen de verschillende studies kunnen nog al eens verschillen worden gevonden. Deze verschillen, zoals bijvoorbeeld in de keuze van een bufferoplossing, kunnen doorslaggevend zijn voor het eindresultaat. Voor het A4502 antilichaam van DAKO wordt dit fraai geïllustreerd op de website van de samenwerkende Scandinavische laboratoria: http://www.nordiqc.org/Assessments/Run7/CD117-as7.htm (oktober 2003).

Voor doeleinden in de oncologie wordt immunohistochemie gebruikt voor classificatie, prognose bepaling en voorspelling van de reactie op therapie. P-gp. MRP1, LRP, TRAIL-receptoren en c-KIT zijn betrokken bij het biologische gedrag van tumoren en kunnen tevens dienen als aangrijpingspunt voor behandeling van weke-delen sarcomen. Dit benadrukt nog eens het belang van een betrouwbare methode van immunohistochemie. Met betrekking tot c-KIT bij GISTen geldt inmiddels is gebleken dat expressie alleen onvoldoende is om de reactie op imatinib te kunnen voorspellen: de aanwezigheid van gen mutaties geeft aanvullende informatie. Daarom dient rekening te worden gehouden met het gegeven dat moleculaire veranderingen van het aangrijpingspunt voor behandeling het effect van therapie kan beïnvloeden. Aanvullende moleculaire technieken zullen in de nabije toekomst een belangrijke bijdrage gaan leveren in de klinische praktijk van weke delen sarcomen.

Systemische behandeling van weke-delen sarcomen: "Oud en nieuw …"

Doxorubicine: nog steeds de moeite waard? Systemische behandeling van maligniteiten wordt toegepast wanneer locale behandeling met chirurgie en radiotherapie niet haalbaar is. Hoewel de meeste studies lokaal-uitgebreide en gemetastaseerde weke-delen sarcomen als een ongeneeslijke situatie beschouwen, toont recent onderzoek dat er wel degelijk een subgroep bestaat die een 5-jaars overleving heeft na doxorubicine-bevattende chemotherapie.[7] Deze opmerkelijke bevinding lijkt het gebruik van doxorubicine te rechtvaardigen bij uitgebreide weke-delen sarcomen. Toch zou het van grote waarde zijn om vooraf díe patiënten te kunnen identificeren die baat hebben bij dergelijke

chemotherapie. Het zou daarom de moeite waard zijn om na te gaan of de goed reagerende tumoren uit deze studie bijvoorbeeld minder (functionele) multidrug resistentie eiwitten tot expressie brengen.

Conventionele middelen anders dan doxorubicine of ifosfamide. Hoewel doxorubicine en ifosfamide de meest gebruikte middelen bij weke-delen sarcomen zijn, is het niet uitgesloten dat ook andere bestaande cytostatica effectief zijn. Van paclitaxel is effectiviteit aangetoond bij angiosarcomen van het hoofd/hals gebied [8], in tegenstelling tot het effect bij andere types weke-delen sarcomen [9], terwijl docetaxel met gemcitabine effectief was bij patiënten met een uterien leiomyosarcoom.[10]

Gegevens met betrekking tot weke-delen sarcomen wijst uit dat liposomaal doxorubicine even effectief is als standaard doxorubicine, met minder bijwerkingen.[11] Deze specifieke toedieningsvorm zou daarom gebruikt kunnen worden bij patiënten, voor wie men onoverkomelijke cardiotoxiciteit van standaard doxorubicine vreest.

Trofosfamide is een oraal middel, verwant aan ifosfamide en cyclofosfamide, dat relatief goed verdragen wordt en actief is bij weke-delen sarcomen.[12-15] Het milde bijwerkingen profiel en de orale toedieningsvorm maken dit middel een interessant cytostaticum, met name bij patiënten boven de 65 jaar.

Ecteinascidine-743. Ecteinascdine-743 (ET-743, trabectedine) is een nieuw ontwikkeld antikanker middel afkomstig van het zee-organisme *Ecteinascidin turbinata*. ET-743 bindt aan het DNA en remt daarmee DNA transcriptie activatie. In vitro onderzoek heeft aangetoond dat ET-743 effectief is tegen weke-delen sarcoom cellijnen van verschillende histologische types.[16] Inmiddels is ook in de klinische situatie de werkzaamheid van ET-743 tegen weke-delen sarcomen aangetoond.[17-19]

Behalve remming van DNA transcriptie blijkt ET-743 ook gericht de activatie van het *mdr1* gen, coderend voor P-gp, te kunnen remmen.[20] Dit biedt de mogelijkheid om P-gp gemedieerde resistentie tegen antikanker middelen te kunnen verminderen met ET-743. Dit mechanisme is aangetoond in een in vitro model, waarbij weke-delen sarcoom cellen voorbehandeld werden met ET-743, nadien meer doxorubicine opnamen en gevoeliger werden voor doxorubicine.[21]

Thiazolidinediones. Thiazolidinediones zijn een klasse van middelen die ligand zijn voor het peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is een hormoon receptor gelegen in de celkern, betrokken bij de

differentiatie van voorlopercellen tot vetcellen. Echter, naast deze fysiologische rol, is PPAR-γ ook betrokken bij de oncogenese van liposarcomen. Wanneer liposarcoom cellen worden blootgesteld aan het thiazolidinedione pioglitazone, dan treedt vetcel differentiatie op.[22] Een beschrijving van drie gevallen van liposarcomen in vivo maakt melding van differentiatie van tumorcellen tot morfologische normale vetcellen na behandeling met het thiazolidinedione troglitazone.[23] Vanwege het (geringe) risico op ernstige leverschade is troglitazone inmiddels van de markt gehaald; het structureel verwante rosiglitazone werd gebruikt in een volgende klinische studie (NCI-G99-1629 van het *National Cancer Institute* in de Verenigde Staten). Van deze studie zijn echter nog geen eindresultaten bekend; wel wordt voortijdig melding gemaakt van het uitblijven van radiologisch meetbare respons. Op dit moment is het te vroeg om de toepasbaarheid bij maligniteiten van thiazolidinediones gecombineerd met andere middelen in te kunnen schatten. Meldingen van een versterkend effect van thiazolidinediones gecombineerd met TRAIL [24,25] en met NSAIDs [26] kunnen nochtans een belofte voor de toekomst inhouden.

Modulatoren van drug-efflux eiwitten. Ondanks veelbelovende resultaten vanuit in vitro experimenten met remmers van drug-efflux pompen (P-gp, MRP1), hebben deze middelen niet voor een doorbraak kunnen zorgen in de behandeling van solide tumoren. Recent hebben Bramwell en medewerkers wel een antitumor effect aangetoond van VX-710 (Biricodar™), een modulator van P-gp en MRP1 activiteit, in combinatie met doxorubicine in aanvankelijk doxorubicine-refractaire weke-delen sarcomen.[27] Deze resultaten behoeven bevestiging in aanvullende studies, met zorgvuldige monitoring van de bijwerkingen. Toch lijkt deze tweede-generatie drug-efflux remmer een belofte in de behandeling van klassieke MDR in weke-delen sarcomen. Inmiddels hebben derde-generatie MDR blokkers klinische studies bereikt.[28]

P-gp is betrokken bij resistentie tegen conventionele antikanker middelen, maar recent onderzoek toont aan dat P-gp ook het effect van nieuwe middelen zoals imatinib mesylaat kan verminderen.[29,30] Het lijkt daarom interessant om te onderzoeken of maligne GISTen gevoeliger worden, of weer gevoelig worden voor imatinib in combinatie met een P-gp remmer. Temeer, omdat juist in maligne GISTen een overmatige expressie van P-gp gevonden wordt.[31]

Remmers van receptor tyrosine kinase activiteit. Signaal transductie is het proces van omzetting van extracellulaire signalen naar een intracellulaire respons. Signaal transductie wordt voor een belangrijk deel geïnitieerd door binding van het ligand aan receptor tyrosine kinases waarmee proliferatie, differentiatie en apoptose worden gereguleerd. Ontregeling van receptor tyrosine kinase activiteit leidt tot verstoorde signaal transductie en kan maligne ontaarding van cellen tot gevolg hebben. De enzymatische functie van receptor tyrosine kinases is gelegen in het intracellulaire domein en omvat de overdracht van een fosfaatgroep van adenosine trifosfaat (ATP) op het specifieke substraat. Deze stap kan geremd worden door klein-moleculaire stoffen die met ATP competeren; deze middelen vormen een geheel nieuwe klasse van antikanker middelen.

Voor de weke-delen sarcomen zijn een aantal receptor tyrosine kinases bekend die mogelijk aangrijpingspunt zijn voor therapie. Voor enkele zijn reeds klein-moleculaire remmers beschikbaar: c-KIT, platelet-derived growth factor (PDGF-R) en vascular endothelial growth factor (VEGF-R).

Als model kunnen de maligne GISTen dienen, waarbij deregulatie van c-KIT tyrosine kinase activiteit bestaat als gevolg van een mutatie in het *c-kit* gen. Imatinib, aanvankelijk ontworpen als remmer van Bcr-Abl tyrosine kinase in chronische myeloïde leukemie, remt tevens c-KIT en PDGF-R. In GISTen heeft imatinib een spectaculair effect.[32] Heinrich et al toonden aan dat dit effect afhangt van de aanwezigheid van activerende mutaties in exon 11 van het *c-kit* gen.[33] Maligne GISTen zonder mutaties van het *c-kit* gen zijn aanzienlijk minder gevoelig voor imatinib.[34] Maar ook 15% van de GISTen mét een exon 11 mutatie vertoont intrinsieke resistentie tegen imatinib. Daarbij komend blijkt dat aanvankelijk gevoelige GISTen resistentie kunnen ontwikkelen tegen imatinib.[35] GISTen die resistent zijn tegen imatinib lijken echter nog wel behandelbaar met nieuwe alternatieve remmers van tyrosine kinases, zoals SU11248.[36] Naast remming van c-KIT, remt SU11248 ook PDGF-R, VEGF-R en FLT3 (fms-related tyrosine kinase 3). Het effect zou dus ook gemedieerd kunnen worden door remming van (één of meerdere van) deze receptor tyrosine kinases.

Resultaten behaald in GISTen hebben ertoe geleid om het effect van imatinib ook voor andere weke-delen sarcomen te onderzoeken. Tot nu toe lijkt imatinib weinig effectief bij andere types weke delen sarcomen, die geen c-KIT tot expressie brengen.[37] Een uitzondering vormt het dermatofibrosarcoma protuberans (DFSP), dat ook op imatinib kan reageren.[38] Deze tumoren worden gekenmerkt door expressie van het COL1A1-PDGF β fusie eiwit, dat autocriene stimulatie van PDGF-R β geeft. Remming van PDGF-R β induceert apoptose van DFSP cellen.[39]

Gegevens uit een xenograft model suggereren dat imatinib ook voor het (extrasketaal) Ewing sarcoom effectief zou kunnen zijn.[40] In dit geval wordt het effect gemedieerd door remming van overmatige c-KIT activiteit, uitgelokt door autocriene stimulatie van het natuurlijke ligand. Gegevens van toepassing van imatinib bij patiënten met een Ewing sarcoom zijn nog niet gepubliceerd.

Het klinische succes van receptor tyrosine kinase remmers zal ongetwijfeld niet beperkt blijven tot imatinib gericht tegen c-KIT in maligne GISTen. Andere remmers zijn in ontwikkeling of zijn al beschikbaar, gericht tegen c-KIT maar ook tegen andere receptor tyrosine kinases. SU6668 bijvoorbeeld, remt VEGF-R, PDGF-R en FGF-R (fibroblast growth factor receptor). VEGF-R activiteit is aangetoond in angiosarcomen en het ligt voor de hand om in dit type weke-delen sarcoom het effect van VEGF-R remming te onderzoeken.[41]

Op dit moment wordt in een EORTC fase II studie (#62022) aan patiënten met synoviosarcomen die de standaardbehandeling reeds gehad hebben, gefitinib (ZD 1839, Iressa™, Astra Zeneca) aangeboden, dat epidermal growth factor 1 (EGF-R1) remt.

De geheel nieuwe klasse van klein-moleculaire remmers van receptor tyrosine kinases brengt ook nieuwe bijwerkingen met zich mee. Over de lange termijn effecten valt nog weinig te melden. De eerste patiënten met imatinib zijn twee tot drie jaar onder behandeling. Ernstige acute bijwerkingen van imatinib zijn tumorbloeding bij GIST patiënten en beenmergdepressie voornamelijk bij chronische myeloïde leukemie patiënten. Een vreemdsoortige bijwerking is de terugkeer van pigment in grijs haar, waarschijnlijk veroorzaakt door modulatie op c-KIT activiteit in melanocyten van haarfollikels.[42]

TRAIL. Tumor necrosis factor-related apoptosis inducing ligand, of TRAIL, herbergt de belofte van effectieve apoptose-inductie in tumor cellen, terwijl normale weefsels worden gespaard. TRAIL kan ook apoptose induceren in mesenchymale tumor cellen; evenwel lijkt de ontwikkeling van TRAIL-resistentie niet uit te sluiten. Wanneer TRAIL klinisch toepasbaar wordt, zal daarom ook rekening worden gehouden met TRAIL-ongevoelige weke-delen sarcomen. In dit proefschrift wordt beschreven dat de combinatie van TRAIL met een conventioneel cytostaticum een manier biedt om TRAIL-resistentie in weke-delen sarcoom cellen te omzeilen. Het zou echter aantrekkelijker zijn om een soortgelijk effect te krijgen door TRAIL met niet-toxische middelen te combineren. In vitro onderzoek toont aan dat niet-steroïdale anti-

inflammatoire middelen (NSAIDs) en thiazolidinediones hier in principe voor in aanmerking komen.[25,43,44]

LRP

Van de eiwitten die betrokken zijn bij MDR, is het meest bekend over de groep van ATP-binding cassette eiwitten, waartoe P-gp en de MRP-familie behoren. Lung resistance-related protein, of LRP, daarentegen neemt een aparte positie in, zowel qua structuur als qua functie; zelfs de oorzakelijke rol van LRP in resistentie is niet eenduidig. Zo leidt transfectie van het LRP gen in tumor cellen niet tot MDR.[45] Kitazono et al leverden als eersten het bewijs voor een causale rol van LRP in het fenomeen van MDR.[46] Echter, tot op heden is het andere onderzoeksgroepen niet gelukt om dit resultaat te bevestigen.[47] Onlangs zijn muizen ontwikkeld die over het intacte LRP gen beschikken, maar desondanks niet toegenomen gevoelig zijn voor de toxische effecten van doxorubicine.[48] Deze resultaten wijzen erop dat LRP niet direct betrokken is bij MDR. Toch lijkt het voorbarig om link tussen LRP en MDR te laten varen, gezien de resultaten van klinisch-pathologische onderzoeken [49-51], alsmede hoofdstukken 4 en 5 van het huidige proefschrift.

Nieuwe middelen, nieuwe eindpunten

Cytotoxische middelen doden tumor cellen, waardoor de grootte van de tumor afneemt. Deze veranderingen in tumor-omvang kunnen gemeten worden met standaard afbeeldingtechnieken (röntgenfoto's, spiraal computer tomografie, magnetische resonantie afbeelding) en vormen de basis voor evaluatie van het antitumor effect. Voor weke-delen sarcomen zijn de RECIST criteria hanteerbaar [52], en in het concept van fase 2 studies bepalen deze criteria of een middel verder onderzoek waard is. Nieuwe, niet-cytotoxische stoffen zoals thiazolidinediones en imatinib hoeven echter niet perse een afname in tumorgrootte te laten zien in geval van een klinische respons. Zo veroorzaakt het thiazolidinedione troglitazone vetcel-differentiatie in liposarcomen [23]; ogenschijnlijk paradoxaal neemt daarmee de tumoromvang toe, omdat tumorcellen onder invloed van troglitazone vetdruppels beginnen te accumuleren. Imatinib mesylaat vertraagd het metabolisme van GIST cellen, en een afname in tumorgrootte hoeft pas na verloop van tijd te volgen.[53] Deze twee voorbeelden illustreren

dat voor niet-cytotoxische middelen zijn alternatieve eindpunten nodig om het antitumor effect te evalueren. Omdat deze middelen de voortgang van de ziekte tegengaan, zou de progressie-arrest ratio gebruikt kunnen worden als surrogaat parameter van activiteit. Middelen die duidelijk invloed hebben op het tumormetabolisme zijn geschikt voor evaluatie met positron emissie tomografie. Daarbij kan een tracer worden gebruikt die betrokken in het metabolisme, zoals ^{18}F-fluorodeoxyglucose.[54] Sequentiële tumor bioptering is een directe methode om de respons op behandeling te kunnen duiden, maar het invasieve karakter ervan stuit op praktische en ethische bezwaren. Serum markers die specifiek zijn voor bepaalde typen weke-delen sarcomen zijn niet voorhanden en hebben dus geen klinische toepassing.

Weke-delen sarcomen zijn intrigerende maligniteiten vanwege hun unieke biologische eigenschappen. Deze zeldzame tumoren kunnen het best benaderd worden via een hierin bekwaamd oncologisch team, bestaande uit clinici en onderzoekers. De huidige behandeling met chirurgie en radiotherapie is helder gedefinieerd en lokale controle is goed.[55] Toch zal ongeveer 30-40% van de patiënten metastasen ontwikkelen. De uitdaging is hiervoor effectieve behandelingen te vinden, liefst natuurlijk met een zo gunstig mogelijk bijwerkingen profiel. Tevens zou een betere selectie van patiënten die adjuvante behandeling na lokale therapie moeten hebben, een grote vooruitgang betekenen. Alleen onderzoek, gewijd aan patiënten met deze ziekte, kan verandering brengen in de huidige vooruitzichten. De doorbraak van imatinib in de behandeling van maligne GISTen dient hierbij als bron van motivatie voor onderzoekers en artsen die werkzaam zijn in het aandachtsgebied van de weke delen sarcomen.

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Stellingen behorend bij het proefschrift van Rudy Komdeur

Resistance and Perspectives in Soft Tissue Sarcomas

1. *Het* weke-delen sarcoom bestaat niet.
2. Verschillen in expressie van multidrug resistentie eiwitten onderschrijven het belang van onderzoek bij weke-delen sarcomen per histologisch type. *(dit proefschrift)*
3. Weke-delen sarcomen zouden aanvullend geclassificeerd moeten worden op aanwezigheid van determinanten van tumorbiologisch gedrag. *(dit proefschrift)*
4. De sterkst gedifferentieerde rhabdomyosarcoom cellen bezitten een verhoogde expressie van het lung resistance-related protein en blijken preferentieel beschermd tegen chemotherapie. *(dit proefschrift)*
5. De uitgebreide expressie van lung resistance-related protein in rhabdomyosarcomen bij volwassenen suggereert een relatie met hun relatieve ongevoeligheid voor chemotherapie. *(dit proefschrift)*
6. Met de goede resultaten van de huidige lokale behandeling van weke-delen sarcomen, zal het onderzoek zich nu moeten richten op het verminderen van de door behandeling geïnduceerde lange termijn morbiditeit.
7. Systemische lekkage van tumor necrosis factor-α en melfalan tijdens hyperthermische geïsoleerde ledemaat perfusies draagt niet bij aan klinisch relevante veranderingen in multidrug resistentie eiwitten in eventuele micrometastasen. *(dit proefschrift)*
8. Naast het uitpompen van xenobiotica, wordt P-glycoproteïne functie geassocieerd met het verhinderen van caspase-gemedieerde apoptose; de route waarlangs tumor necrosis factor-α en TRAIL hun effect sorteren.
9. Doxorubicine versterkt in vitro het apoptose-inducerend vermogen van TRAIL. *(dit proefschrift)*
10. Pigmentatieveranderingen door imatinib hebben zowel positieve als negatieve invloeden op de therapietrouw van het gebruik van dit geneesmiddel.
11. De routekaart naar de vrede heeft geen schaalverdeling.